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ACADEMIC DISSERTATION
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Responsible supervisor and Custos	Professor Marko Pesu Tampere University Finland	
Pre-examiners	Professor Tom Pettersson University of Helsinki Finland	Docent Arno Hänninen University of Turku Finland
Opponent	Professor Kunihiro Yamaoka Kitasato University Japan	

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"Dreaming, after all,
is a form of planning."

- Gloria Steinem (1934-)

ABSTRACT

The prevalence of immune-mediated diseases is rising rapidly in our aging population. Currently used biomarkers are inaccurate and have a low predictive value, which complicates early diagnosis and evaluation of the efficacy of a given treatment. Consequently, there is an urgent need for novel biomarkers for immune-mediated diseases at present. Proprotein convertase subtilisin/kexin (PCSK) enzymes have an important role in regulating general homeostasis by dictating the bioavailability of a variety of secretory and cell-surface proteins. The first found PCSK enzyme Furin is readily upregulated during immune cell activation. It regulates T cell functions and induces the processing of toll-like receptor 7 in innate immune cells to promote host defense against intracellular and extracellular pathogens. Furthermore, T-cell-expressed Furin favors T helper 1 cell polarization and promotes proper forkhead box P3+ T regulatory cell functions. Hence in addition to its key regulatory role in innate and adaptive immune responses, Furin is crucial for the maintenance of peripheral immunological tolerance. On the contrary, alterations in Furin expression have been associated with several immune-mediated diseases.

This study investigated the role of Furin expression in acute infection, primary Sjögren's syndrome and rheumatoid arthritis using various detection methods. The main aim was to assess how Furin levels correlate with clinical parameters in order to study its potential value as a biomarker for the aforementioned immune-mediated diseases. The results show that the Furin plasma level does not identify severe infections or initial pathogens in the early phase of infections. However, Furin expression was significantly elevated in both primary Sjögren's syndrome and rheumatoid arthritis. Elevated Furin levels were associated with high interferon-γ levels and prolonged symptoms of dry eye in the group of patients with primary Sjögren's syndrome. Patients with high Furin levels also showed a trend toward lower β2-microglobulin levels, erythrocyte sedimentation rate, and systemic disease activity index ESSDAI. In contrast, Furin plasma levels were not associated with clinical parameters in patients with rheumatoid arthritis. However, elevated Furin messenger ribonucleic acid levels were significantly associated with prednisolone use

and prednisolone doses, C-reactive protein levels, and general disability index (Health Assessment Questionnaire).

Collectively, this study shows that Furin plasma measurements do not have a predictive or diagnostic value in the early phase evaluation of patients with suspected infection. On the contrary, Furin was significantly upregulated in both primary Sjögren's syndrome and rheumatoid arthritis, suggesting that it may have a role in the pathogenesis of autoimmune diseases. However, the observations of Furin effects turned out contradictory: Results from the primary Sjögren's syndrome group suggested its protective role in the disease development, whereas elevated levels in rheumatoid arthritis indicated a more severe and refractory disease. This discrepancy could be a result of its extensive substrate selection. Furin is known to activate both proinflammatory and anti-inflammatory factors, which could lead to its varying effects in terms of distinct pathophysiological mechanisms. Additional studies and novel methods for the detection of Furin are needed to further evaluate the clinical relevance of Furin expression in predicting immune-mediated diseases and understand its fundamental nature in health and disease.

TIIVISTELMÄ

Immuunivälitteiset sairaudet ovat yleistyvä ongelma väestön ikääntyessä. Tällä hetkellä käytössä olevat biomarkkerit ovat kuitenkin epätarkkoja ja ennustearvoltaan heikkoja, minkä vuoksi tämän potilasryhmän varhainen tunnistaminen ja tehokas hoito on osoittautunut haastavaksi. Proproteiinikonvertaasi subtilisiini/keksiini (PCSK) entsyymit kontrolloivat useiden, solujen tuottamien proteiinien bioaktiivisuutta ja ovat siten keskeisiä elimistön homeostasian säätelytekijöitä. Furin on eniten tutkittu PCSK-entsyymi ja sen ilmentyminen on todettu lisääntyvän erityisesti immuunijärjestelmän aktivaatiossa. Furin edistää toll-like reseptori 7:n prosessointia luonnollisen immuunivasteen soluissa sekä säätelee T-auttajasolujen toimintaa suosimalla T_H1-tyyppistä immuunivastetta. Lisäksi sen ilmentyminen T-auttajasoluissa voimistaa forkhead box P3 positiivisten T-säätelijäsolujen tulehdusta hillitsevää toimintaa ja rajoittaa siten liiallista tulehdusreaktiota. Furin on keskeinen tekijä sekä luonnollisen että hankitun immuunivasteen säätelyssä ja sen poikkeava ilmentyminen on yhdistetty immuunivasteen toiminnan häiriöihin sekä useisiin immuunivälitteisiin sairauksiin. Tästä huolimatta sen perimmäinen rooli immuunijärjestelmän toiminnassa tunnetaan edelleen puutteellisesti. Näin ollen tutkimuksia Furinin käyttömahdollisuuksista kliinisessä työssä on vasta vähän.

Tässä väitöskirjassa tutkittiin Furinin ilmentymistä akuutissa infektiossa, primaarissa Sjögrenin syndroomassa ja nivelreumassa hyödyntäen erilaisia mittausmenetelmiä ja kliinisiä potilasaineistoja. Päätavoitteena oli selvittää Furin-mittausten käytettävyyttä infektiotautien biomarkkerina päivystyspoliklinikalla sekä niiden kliinistä relevanssia edellä mainittujen autoimmuunisairauksien diagnostiikassa, hoidon suunnittelussa ja seurannassa. Tulosten perusteella Furinin plasmapitoisuus ei ennusta kuolleisuutta eikä tunnista bakteeritulehdusta infektiopotilaiden päivystyksellisen arvioinnin yhteydessä. Sen sijaan plasman Furin-pitoisuudet olivat merkitsevästi korkeampia potilailla, joiden sairaushistoriasta löytyi diagnosoitu reumatauti. Tutkimuksissamme Furinin ilmentyminen osoittautui selvästi kohonneeksi sekä primaarissa Sjögrenin syndroomassa että nivelreumassa. Primaarissa Sjögrenin syndroomassa korkea Furin-plasmapitoisuus oli yhteydessä korkeisiin interferoni-γ tasoihin sekä pidempikestoisiin kuivasilmäisyysoireisiin. Lisäksi havaittiin trendi, jossa lasko, systeeminen tautiaktiivisuusindeksi ESSDAI ja

seerumin β2-mikroglobuliini olivat keskimäärin matalampia potilailla, joiden plasman Furin oli korkea. Sen sijaan Furinin plasmapitoisuudet eivät liittyneet kliinisiin parametreihin nivelreumapotilaista koostuvassa aineistossa. Kuitenkin Furinia koodaavan lähetti-RNA:n lisääntynyt pitoisuus perifeerisen veren mononukleaarisissa soluissa liittyi merkitsevästi kortisonilääkityksen tarpeeseen ja kortisoniannokseen, koholla olevaan C-reaktiiviseen proteiiniin sekä toiminnanvajeeseen toimintakykykyselyssä (Health Assessment Questionnaire).

Tämä väitöskirja osoitti, että Furin ei sovellu infektiomittariksi päivystyspoliklinikalla eikä sen plasmapitoisuudesta voida tehdä johtopäätöksiä liittyen taudin vaikeusasteeseen tai potilaan ennusteeseen. Lisäksi Furinin pitoisuutta plasmassa ei voida hyödyntää empiirisen antibiootin valinnassa taudin alkuvaiheessa. Toisaalta Furinin ilmentymisen todettiin lisääntyvän merkitsevästi sekä primaarissa Sjögrenin syndroomassa että nivelreumassa. Tulokset viittasivat Furinin mahdollisesti suojaavaan vaikutukseen primaarissa Sjögrenin syndroomassa, kun taas nivelreumassa korkea Furin-taso näytti liittyvän aktiivisempaan ja hoitoresistenttiin taudinkuvaan. Tutkimuksissa tehtyjen löydösten perusteella Furinin toiminta ja ilmentyminen saattaa olla merkityksellistä autoimmuunisairauksien patogeneesissä. Furin käyttäytyy mahdollisesti tautispesifisesti johtaen joko aktiivisempaan (nivelreuma) tai lievempään (primaari Sjögrenin syndrooma) taudinkuvaan riippuen taudin taustalla olevasta patogeneesistä. Tämä voisi selittyä Furinin laajalla substraattivalikoimalla. Koska Furin aktivoi sekä immuunivastetta hillitseviä että kiihdyttäviä tekijöitä, erilaiset patomekanismit voisivat vaikuttaa Furinin rooliin immuunijärjestelmän toiminnassa. Jatkotutkimukset ovat välttämättömiä Furinin toiminnan ymmärtämisessä sekä arvioitaessa sen kliinistä käytettävyyttä immuunivälitteisten sairauksien diagnostiikassa sekä hoidon seurannassa. Uudet mittausmetodit saattavat olla ratkaisevassa roolissa avaten uusia mahdollisuuksia hyödyntää Furinia kliinisessä päätöksenteossa.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referenced in the text by their Roman numerals (I–III):

I **Ranta N**, Turpeinen H, Oksanen A, Hämäläinen S, Huttunen R, Uusitalo-Seppälä R, Rintala E, Aittoniemi J, Pesu M. The Plasma Level of Proprotein Convertase FURIN in Patients with Suspected Infection in the Emergency Room: A Prospective Cohort Study. *Scand J Immunol* 2015;82(6):539-546.

II **Ranta N*,** Valli A*, Grönholm A, Silvennoinen O, Isomäki P, Pesu M*, Pertovaara M*. Proprotein convertase enzyme FURIN is upregulated in primary Sjögren's syndrome. *Clin Exp Rheumatol.* 2018;36 112(3):47-50.

III Valli A*, **Ranta N*,** Grönholm A, Silvennoinen O, Pesu M*, Isomäki P*. Increased expression of the proprotein convertase enzyme FURIN in rheumatoid arthritis. *Scand J Rheumatol.* 2019; 48(3):173-177.

*) Equal contribution

ABBREVIATIONS

ACPA	autoantibodies against citrullinated protein antigens
AIRE gene	autoimmune regulator gene
anti-CarP	autoantibodies against carbamylated proteins
anti-La/SSB	anti-La/Sjögren's syndrome antigen A
anti-PAD	autoantibodies against peptidyl arginine deiminases
anti-Ro/SSA	anti-Ro/Sjögren's syndrome antigen A
APC	antigen presenting cell
BAFF	B cell activation factor
BCR	B cell receptor
CLP	common lymphoid progenitor
CRP	C-reactive protein
DAMP	damage-associated molecular pattern
DC	dendritic cell
DMARD	disease-modifying antirheumatic drug
EULAR	European League Against Rheumatism
ER	emergency room
ESR	erythrocyte sedimentation rate
ESSDAI	EULAR Sjögren's syndrome disease activity index
ESSPRI	EULAR Sjögren's syndrome patient reported index
FoxP3	forkhead box P3
IC	immune complex
FUR	*FES* upstream region
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILC	innate lymphoid cell
IMD	immune-mediated disease
iT_{reg} cell	inducible T regulatory cell
LPS	lipopolysaccharide
MAP	mean arterial pressure

MC	mononuclear cell
MHC	major histocompatibility complex
MOF	multi-organ failure
mRNA	messenger ribonucleic acid
NK cell	natural killer cell
NLR	NOD-like receptor
nT_{reg} cell	natural T regulatory cell
PAMP	pathogen-associated molecular pattern
PB	peripheral blood
PCSK	proprotein convertase subtilisin/kexin
PRR	pattern recognition receptor
pSS	primary Sjögren's syndrome
Q-RT-PCR	quantitative real-time polymerase chain reaction
qSOFA	quick sequential organ failure assessment
RA	rheumatoid arthritis
RF	rheumatoid factor
RNA	ribonucleic acid
SF	synovial fluid
SIRS	systemic inflammatory response syndrome
SLE	systemic lupus erythematosus
SOFA	sequential organ failure assessment
SS	Sjögren's syndrome
sSS	secondary Sjögren's syndrome
STAT	signal transducer and activator of transcription
TBP	TATA-binding protein
TCR	T cell receptor
T_{FH} cell	follicular T helper cell
TGF-β	transforming growth factor β
T_H cell	T helper cell
TLR	Toll-like receptor
TNF	tumor necrosis factor
T_{reg} cell	T regulatory cell

1 INTRODUCTION

The human immune system is a complex network that provides defense against infectious pathogens including viruses, bacteria, parasites and fungi. In addition, it disposes deregulated host cells and cell debris to maintain cellular homeostasis. The immune system is traditionally divided into two subsystems known as innate and adaptive immune systems. Innate immunity is characterized by fast and nonspecific acts against commonly encountered invaders, whereas adaptive immunity is able to supply relatively slow, but highly specific, effector functions and has long-lasting features. The immune system consists of primary and secondary lymphatic organs, initial barriers, immune cells, and a vast number of soluble immune mediators. Because of comprehensive cellular signaling, the aforementioned subsystems are able to complement each other and produce an effective host response against various internal and external threats. However, dysregulated host defense can be detrimental and lead to severe health problems, such as immunodeficiency, allergies, and autoimmunity. These are generally referred to as immune-mediated diseases (IMDs) and are characterized by tissue damage caused by incorrectly operating immune cells and cytokine dysregulation.

IMDs are an increasing health concern in our aging population. It is estimated that rheumatoid arthritis (RA) alone affects around 1% of all adults, putting a significant economic burden on our society. In addition, the symptoms cause functional disability and result in a notable decrease in the measured quality of life. Despite intensive research work, the underlying factors in the lost control of immune response remain elusive. As a consequence, the development of effective diagnostic tools and targeted treatment methods has become a challenge. Combinations of symptoms, clinical signs, laboratory tests, imaging and biopsies are currently used to diagnose IMDs. The requirement of disease characteristic symptoms as a diagnostic criterion is problematic because they tend to reflect irreversible tissue damage. Hence, the optimal time for treatment is often missed. In addition, the absence of one biomarker to confirm the diagnosis causes delayed, or even missed, detection of patients with IMD. These together result in abundant functional disability and a loss in quality of life.

Multiple laboratory tests are currently used to evaluate immune functions in order to improve timely diagnosis of IMDs. For example, serum autoantibodies against citrullinated protein antigens (ACPA), rheumatoid factor (RF; immunoglobulin M, IgM or IgA) and traditional markers for acute phase response (erythrocyte sedimentation rate, ESR and C-reactive protein, CRP) are considered the most important laboratory tests for RA diagnosis. In addition, novel biomarkers such as autoantibodies against carbamylated proteins (anti-CarPs) and autoantibodies against peptidyl arginine deiminases isoforms (anti-PADs) are recently reported and might aid clinical decision-making in the future. However, all of the aforementioned markers can also be found during adequate immune functions. In other words, they all lack sufficient sensitivity for immune dysregulation. New biomarkers are hence urgently needed to improve early detection of IMDs. Moreover, a better understanding of the underlying pathogenesis is required to develop specific treatment strategies that would match with the particular pathophysiological profile.

Proprotein convertase subtilisin/kexin (PCSK) enzymes regulate general homeostasis by dictating the bioactivity of several secretory proteins. Furin is the first identified, and hence best studied, PCSK enzyme and has been shown to be predominantly upregulated during immune activation. Both adaptive and innate immune cells express Furin, but its fundamental regulatory role in immune response continues to be poorly understood. Lipopolysaccharide (LSP) – a characteristic component of gram-negative bacteria – is known to stimulate Furin expression in acute infection, where it enhances toll-like receptor processing and promotes T helper (T_H) cell polarization toward T_H type 1 response. In addition, T_H-cell-expressed Furin has been proved to play an essential role in the maintenance of immunological tolerance, as it regulates the functional maturity of anti-inflammatory transforming growth factor beta 1 (TGF-β1) and is critical for the development of T regulatory (T_{reg}) cells. The fact that Furin is secreted from activated immune cells makes it relatively easy to measure. This has pointed out an interesting aspect considering the use of Furin measurements in clinical practice. Its altered expression has previously been associated with several pathological conditions, such as chronic inflammation in atherosclerosis, aggressive metastatic cancer, and Alzheimer's disease. However, its potential value as a biomarker for IMDs has not yet been evaluated.

The main aim of this doctoral thesis is to investigate the potential role of Furin as a biomarker for three IMDs, including acute infection, primary Sjögren's syndrome (pSS), and RA. This research is directly focused on estimating its usability

in diagnostics, assessing the disease activity, and evaluating the prognosis and treatment response.

2 REVIEW OF THE LITERATURE

2.1 The innate immune system

Innate immunity is the most primitive unit of immunity and is present in every plant and animal (Flajnik & Kasahara, 2010). It is characterized with its rapid response and operation in first-line defense against commonly encountered pathogens. Innate immune cells are also essential in the clearance of nonfunctional particles, such as cellular debris and damaged or dysregulated host cells. By maintaining a convenient milieu for cell function and proliferation it promotes the survival of appropriately operating cells. It is worth noting that innate immunity lacks the ability for specificity and sustainability, which results in limited acts in host defense. Activation of innate immunity leads to similar functions in spite of the initial activator, and these acts cannot be enhanced during lifetime (Murphy & Weaver, 2017).

Since we are constantly surrounded by various potentially life-threatening microorganisms, the most beneficial method to avoid pathogen invasion is to deny their access in the very beginning. The initial barriers include physical, chemical, and biological shields that are together responsible for providing sufficient barrier permeability. Skin and mucosa form a physical fence, fatty acids create a disadvantageous milieu for proliferation, and microorganisms of normal flora act as natural competitors for intruders, for example. Consequently, only a fraction of external threats are able to enter the body in the first place (Elias, 2007; Murphy & Weaver, 2017). However, even when pathogens are successful in breaking the first-line barriers, they rarely manage to cause significant host tissue damage. This is due to innate immune cells and the highly organized complement system, which provide rapid second-line defense if necessary. They reside in tissues or circulate in the bloodstream to provide a comprehensive detection of any imbalance in general homeostasis. They also play a key role in the initial battle against internally generated threats, for example, deregulated tumor cells that could easily take over tissues if not suppressed. Once activated, additional innate units are recruited at a great rate to strengthen this second-line immune response in the infection site (Rivera, Siracusa, Yap, & Gause, 2016).

One of the most significant facilities in providing accurate immune functions is the ability to distinguish self from nonself and functional from nonfunctional particles. Innate immune cells express nonspecific pattern recognition receptors (PRRs) that recognize general danger signs, including pathogen- and damage-associated molecular patterns (PAMPs and DAMPs). PAMPs are present on the surface of common invasive microbes, whereas DAMPs are released from host cells during cell stress or cell death (Akira, Uematsu & Takeuchi, 2006; Coers, 2013; Matzinger, 1994) . Human PRRs are further classified into several receptor types in accordance with their location and detection specialties. For example, toll-like receptors (TLRs) represent a class of transmembrane receptors, whereas nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are expressed in the cell cytoplasm (Franchi, Warner, Viani, & Nuñez, 2009; Kawai & Akira, 2010). In general, PRR ligation causes changes in receptor structures, which initiates intracellular signaling and eventually results in innate immune cell activation (Akira et al., 2006; Coers, 2013; Matzinger, 1994). It is worth noting that innate immune cells represent limited detection capacity because of their lack of specific features. They recognize common pathogens and damaged host cells but cannot provide antigen-targeted immune functions or immunological memory. Consequently, adaptive immunity is often needed to eliminate invaders, expressing more advanced defense mechanisms. For example, Mycobacterium tuberculosis and Chlamydia trachomatis are able to survive and replicate inside host cells where they cannot be found by innate immune cells (Byrne & Ojcius, 2004; McDonough, Kress, & Bloom, 1993). In these circumstances, adaptive immunity is essential in complementing immune acts to secure effective host defense (Joffre, Nolte, Spörri, & Sousa, 2009).

The complement system is a highly organized network comprising a number of soluble proenzymes. It is designed to enhance both innate and adaptive immune functions and operates in both extracellular and intracellular space. Its soluble units are organized into an amplifying proteolytic cascade where every activated enzyme activates several new proenzymes. The activation can be induced by classical, alternative, or lectin pathway, each leading to the generation C3 convertase and formation of active components C3a and C3b. The final effector functions include pathogen tagging via opsonization (C3b), formation of membrane attack complex (C5b-9), release of anaphylatoxins (C3a, C4a and C5a), and finally the activation of adaptive immune responses. However, it is noteworthy that complement activation is important also during normal homeostasis as it constantly detects and silently eliminates apoptotic cells and cell debris without activating other immune components (Merle, Church, Fremeaux-Bacchi, & Roumenina, 2015).

2.1.1 The cells of the innate immune system

Innate immune cells include phagocytes, granulocytes, natural killer (NK) cells, and innate lymphoid cells (ILCs) (Kvell, Cooper, Engelmann, Bovari, & Nemeth, 2007; Murphy & Weaver, 2017; Sonnenberg & Artis, 2015). They are specialized to identify and eliminate distinct pathogen types by expressing various effector mechanisms. All immune cells are originally derived from hematological stem cells in bone marrow. However, the later division into different maturation pathways explains their diverse functions in host defense. The division into myeloid and lymphoid lineages occurs in the very early phase of maturation. Myeloid progenitors give rise to phagocytes and granulocytes, whereas NK cells and ILCs originate from lymphoid stem cells simultaneously with adaptive immune cells, hence expressing rather lymphocyte-like actions (Fogg et al., 2006; Kvell et al., 2007; Sonnenberg & Artis, 2015).

Phagocytes are specialized to eliminate foreign fragments by engulfing and dissolving them in their intracellular vesicles. These cells are further classified into macrophages, neutrophils, and dendritic cells (DCs) according to their special mode of operation in immune defense (Murphy & Weaver, 2017). Macrophages lodge in tissues and are often described as general scavengers, forming the basis for cellular innate immunity. They either reside in common colonization sites or circulate in the bloodstream to enable fast detection of any imbalance in general homeostasis. They are important not only in the clearance of pathogens but also in the elimination of abundant host material in noninfectious stages (Wynn, Chawla, & Pollard, 2013). M1 macrophages are mainly upregulated during pathogen invasion and characterized by proinflammatory functions, whereas M2 macrophages rather suppress inflammation and participate in tissue repair. After the detection of pathogens, activated M1 macrophages secrete chemotactic molecules to attract other immune cells to the infection site (Gordon, Plüddemann, & Martinez Estrada, 2014).

Neutrophils are known for providing the first-line cellular defense and have a pivotal role in limiting infection in the very beginning. They are present in the bloodstream in large quantities and, hence, can quickly amplify innate immune responses after being recruited from surrounding vessels (Nathan, 2006).

Other granulocytes include eosinophils, basophils and mast cells. These cells can be distinguished because of their numerous cytoplasmic granules. The identification of intruders stimulates intracellular vesicles and the plasma membrane to fuse. As a result, various toxic enzymes are release into the extracellular space. That is to say, granulocytes are specialized to destroy extracellular pathogens too large for phagocytosis (Stone, Prussin & Metcalfe, 2010).

DCs have an important role in enhancing innate immune responses, but because of their relatively slow migration, their functions are more significant in the later stages of infection. DCs are essential in mediating signaling between the innate and adaptive immune systems. After engulfing pathogens, they can display pathogen-specific structures, which are referred to as antigens, in their distinctive cell-surface proteins. Next, they migrate to secondary lymphatic organs to activate naive adaptive immune cells. DCs also participate in defining the nature of the initiated adaptive immune response by secreting cytokines that coordinate T cell differentiation. This ensures the generation of optimal adaptive defense against a particular microbial attack. Because of their regulatory functions, DCs are also known as professional antigen-presenting cells (APCs; Joffre, Nolte, Spörri, & Reis e Sousa, 2009).

NK cells and the recently identified helper-type group 1 (ILC1), group 2 (ILC2), and group 3 cells (ILC3) are referred to as innate lymphoid cells (ILCs), and they differ markedly from other innate immune cells. They originate from lymphoid lineage and resemble adaptive lymphocytes in structure and production of cytokines. Nevertheless, because of the lack of antigen-specific receptors ILCs are still classified as a part of the innate immune system (Kvell et al., 2007). NK cells circulate in the bloodstream and are specialized to recognize and eliminate cells missing general "self"-markers, which are also known as major histocompatibility complexes (MHCs; Vivier et al., 2011). MHC class I molecules are found on the cell surface of every nucleated cells, whereas MHC class II molecules are only present on DCs, macrophages, and B cells. Because of the extensive polymorphism and polygenic trait of MHC genes, all organisms express their individually structured MHCs. They are used to distinguish host cells from foreign and potentially harmful cell types (Murphy & Weaver, 2017). When NK cells detect a distinct or decreased number of MHC class I molecules, they destroy these cells using direct cytotoxicity. Hence, NK cells are often described as the innate version of adaptive cytotoxic T cells (Vivier et al., 2011). In contrast, ILC1, ILC2, and ILC3 are specialized to amplify the surrounding cell signaling in order to strengthen the innate response. They are often found residing in colonization sites, such as the lungs, gut, and dermis, rather than circulating in the bloodstream. They orchestrate early phase immune response and are suggested to provide initial T_H functions during the generation of complete adaptive immune response (Artis & Spits, 2015).

2.2 The adaptive immune system

The adaptive immune system was developed in jawed predators roughly 500 million years ago as a competitive advantage to meet the demands of abundant pathogen supply. Because of evolutionary aspects, adaptive immunity is present only in vertebrates (Flajnik & Kasahara, 2010). It is characterized by highly destructive antigen-targeted functions and has the ability to generate long-lasting protection. In general, the adaptive immune system can be described as a competent host defense backup system for innate immunity. Notable, adaptive immune response is relatively slow and takes rather days, or even weeks, to fully establish. This underlines the importance of innate immune functions in the first-line defense (Murphy & Weaver, 2017).

T and B lymphocytes are the main operators of adaptive immune response. They both originate from hematopoietic stem cells in the bone marrow but differ in terms of further maturation and final effector functions. Activated B cells differentiate into short-lived plasma cells or long-lived memory: both are specialized to express and secrete antigen-specific antibodies (Pieper, Grimbacher, & Eibel, 2013). In contrast, activated T cells provide fairly distinct effector mechanisms on the basis of the T cell subset. Cytotoxic CD8+ T cells act as direct cell-to-cell killers and facilitate host defense against deregulated tumor cells and intracellular pathogens. CD4+ TH cells, in turn, differentiate into various effector subgroups depending on the current tissue microenvironment and epigenetic factors. They are responsible for orchestrating responses of both innate and adaptive immune cells. (Pearce, 2003; Zhu, Yamane, & Paul, 2010)

The ability to differentiate into T and B memory cells enable long-lasting host defense and faster responses in reinfections. As a consequence, the adaptive immune system can improve its defense capacity to match with the present microbial supply during lifetime. (Murphy & Weaver, 2017). It is utilized in vaccinations, where lifelong resistance against well-known harmful, or even lethal pathogens, is generated artificially (Bevan, 2011; Pulendran & Ahmed, 2011).

Lymphocytes are activated trough cell-surface antigen receptors TCRs and BCRs. In contrast to innate PPRs, adaptive recognition receptors are specific to only one particular antigen structure. Because of the wide polymorphism of receptor genes and random rearrangement of receptor structures, the final receptor loci can vary endlessly. Ultimately, the generated repertoire of adaptive recognition receptors is able to detect any antigen (Murphy & Weaver, 2017; Pieper, Grimbacher, & Eibel, 2013). However, antigen receptor ligation is not sufficient alone to trigger

lymphocyte activation and differentiation. Instead, a simultaneous second signal is needed to amplify this initial TCR/BCR signaling. Costimulatory receptors, CD40 for B cells and CD28 for T cells, are located on cell surfaces of cells designed to facilitate lymphocyte activation to ensure definite need for activation. The costimulatory ligands for CD28, CD80 and CD86, are predominantly found on cell surfaces of professional APCs. Correspondingly, the counterparts for CD40 is present only on TH cell surfaces. Therefore TH cells are essential in the generation of effective humoral response (Dong et al., 2001; Murphy & Weaver, 2017; Schoenberger et al., 1998).

2.2.1 T cell development

After the division into myeloid and lymphoid lineages in the bone marrow, a group of common lymphoid progenitors (CLPs) migrate to the thymus for further maturation. The altered nature of signaling promotes encoding of T cell genes and results in the generation of TCR chains and T-cell-type cell surface markers including CD3, CD4 and CD8 (Kondo, Scherer, King, Manz, & Weissman, 2001). After series of sequential changes, all pre-T cells end up expressing CD4 and CD8 on their cell-surfaces. The double-positive cells (CD4+ and CD8+ T cells) migrate into the corticomedullary junction to undergo receptor testing through the mechanisms of central tolerance (Shortman & Wu, 1996).

Thymic epithelial cells express plenty of MHC molecules and are responsible for the initial antigen presenting during the positive selection. Coreceptor stimulation promotes its own transcription and displaces the other coreceptor type. As a result, two single-positive T cell lines are generated; CD4+ T_H cells and CD8+ cytotoxic T cells. Ultimate double positivity indicates nonfunctional surface receptors and these cells are guided into apoptosis via clonal deletion (Murphy & Weaver, 2017; Yamane & Paul, 2013; Zhu, Yamane, & Paul, 2010). After the positive selection the generated single-positive T cells move into the thymic medulla to undergo tests for autoreactivity. Thymic medullary cells encode the autoimmune regulatory (*AIRE*) gene to produce common self-peptides and display them to developing T cells. All T cells with high affinity TCRs to the presented autoantigens are deleted in the mechanisms of negative selection. Finally, single-positive T cells passing both positive and negative selection are hauled to the bloodstream (Abramson, Giraud, Benoist, & Mathis, 2010; Yamane & Paul, 2013).

2.2.2 T cell function

Naive T cells circulate between lymphatic organs to seek out their specific antigen. In general, CD4+ T_H cells detect antigen-MHC class II complexes presented by APCs whereas CD8+ cytotoxic T cells recognized antigens bind to MHC class I molecules presented by several cell types (Miles, McCluskey, Rossjohn, & Gras, 2015). TCR signaling combined with amplifying TCR associated co-receptor signaling leads to induction of T cell activation. Activated lymphocytes proliferate and differentiate into distinct effector T cells in guidance of the strength of TCR signaling, the surrounding cytokine environment and epigenetic regulation (Yamane & Paul, 2013).

Activated DCs and CD4+ T cells provide the required second signals for CD8+ T cells and, hence, regulate the generation of cytotoxic T cell response. Once activated, CD8+ T cells undergo excessive proliferation and develop their characteristic effector functions. These include, for example, the activation of FAS-ligand-mediated apoptosis, secretion of toxic enzymes such as perforin and granzyme B, and production of interferon gamma (IFN-γ), which inhibits viral replication and stimulates macrophage functions in order to promote more effective pathogen clearance (Pearce et al., 2003; Topham, Tripp & Doherty, 1997). (Chang, Wherry & Goldrath, 2014).

On the contrary, only cells that express MHC class II molecules and the mandatory TCR signal amplifiers, CD86 and CD80, are able to activate CD4+ T cells. These cells include macrophages, DCs and B cells of which are, hence, referred to as professional APCs (Miles et al., 2015; Murphy & Weaver, 2017). Even though B cells and macrophages can equally initiate CD4+ T cell activation, DCs are stated as the most Hence initial PRR signaling guides DC actions in order to achieve maturation of the most needed CD4+ T cell subset regarding the particular attacker type. Finally, mature T_H cells leave secondary lymphatic organs and migrate to their specific operating sites (Yamane & Paul, 2013; Zhu et al., 2010).

T_H1 response is generated in the presence of IL-12 and IFN-γ via signal transducer and activator of transcription 1/signal transducer and activator of transcription 4 (STAT1/STAT4)-mediated signaling pathways and is designed to enforce immune responses against intracellular pathogens. T_H1 cells produce IFN-γ, which promotes macrophage activation. In addition, T_H1 cells guide antibody class switching toward opsonizing IgG-type antibodies to result in even more effective phagocytosis (Szabo, Sullivan, Peng, & Glimbcher, 2003).

T_H2 response, in turn, is generated via the IL-4/STAT6 pathway to strengthen immune defense against extracellular invaders too large for engulfment such as parasites. T_H2 cells enforce immunity especially in mucosal surfaces, where they secrete IL-4, IL-5, and IL-13 to lure and activate circulating granulocytes. In addition, T_H2 cells favor IgE class switching to boost granulocyte functions (Yamane & Paul, 2013; Zhu et al., 2010).

The third main T_H-cell-type response is generated in the presence of IL-6, IL-23 and TGF-β via the STAT3 signaling pathway to promote efficient clearance of extracellular pathogens in common barrier sites such as the gut, lungs, and urogenital tract. Mature T_H17 cells produce IL-17 and IL-22, which attract neutrophils, stimulate the local epithelial cells to produce antimicrobial peptides, and prevent further pathogen invasion. In addition, T_H17-cell-type response favors IgG class switching and supports effective pathogen detection (Crome, Wang, & Levings, 2010; Zuniga, Jain, Haines, & Cua, 2013).

T_{FH} and T_{reg} cells develop alongside all previously described T_H cells but differ greatly in terms of their fundamental functions in host defense. T_{FH} cells are mandatory in generating T-cell-dependent antibody responses in germinal centers (GCs). Their maturation comprises multiple stages and involves several promoting factors including IL-6, IL-21, inducible T cell co-stimulator (ICOS), and TCR signaling. Expression of the transcription factor B-cell lymphoma 6 (Bcl-6) and cell-surface C-X-C motif chemokine receptor type 5 are considered hallmarks of T_{FH} cell polarization and play a mandatory role in T_{FH} cell migration (Crotty, 2014). Unlike other T cell types, mature T_{FH} cells stay in secondary lymphatic nodes. They move to B cell zones to provide costimulatory signaling for B cells through CD40 ligation; facilitate B cell differentiation, proliferation, and survival in GCs; and produce B-cell-stimulating cytokines such as IL-21 and IL-4 (Deng, Wei, Fonseca, Graca, & Yu, 2019). T_{FH} cells are essential for the generation of effective B cell functions trough the GC formation and affinity maturation of antibodies (Murphy & Weaver, 2017; Vinuesa, Linterman, Yu, & MacLennan, 2016).

T_{reg} cells, in contrast, are important in providing suppressor functions. They rather restrain than promote inflammation and have a key role in preventing exaggerated immune responses. Natural T regulatory (nT_{reg}) cells are formed in the thymus as part of positive and negative selection, and they represent a group of T cells with the highest affinity for self-antigens still able to pass negative selection criteria. In addition to nT_{reg} cells, DCs assist differentiation of induced T regulatory (iT_{reg}) cells via STAT5-mediated pathway in the presence of IL-2 and TGF-β to provide better control for erroneous immune cell functions in periphery (Pieper et

al., 2013). The ability to express transcription factor forkhead box P3 (FoxP3) in response to self-antigens has been reported as the determining factor in protective functions of T_{reg} cells (Fontenot et al., 2005). In general terms, T_{reg} cells limit inflammation by producing anti-inflammatory IL-10 and TGF-β that prevent antigen presentation and inhibit costimulatory signaling in order to restrain the activation and enforcement of other T_H cell responses. They also express high-affinity IL-2 receptors and hence restrict general availability of IL-2, a significant inducer of T_H cell activation (Josefowicz, Lu, & Rudensky, 2012; Maynard et al., 2007).

Moreover, recent studies have reported new T_H cell subgroups including T_H9 and T_H22 cells, which both are suggested to participate in protective immunity. However, the fundamental role and operation of these new T_H cell types remain unclear (Raphael, Nalawade, Eagar, & Forsthuber, 2015).

In conclusion, professional APCs are essential in regulating the activation of T cells and initiation of a cell-mediated immune response. DCs produce a selection of cytokines to reflect encountered invaders in order to guide T_H cell polarization toward the most suitable T_H cell type (Joffre et al., 2009). Mature T_H cells orchestrate innate and humoral responses through their cytokine secretion. They enforce the prevalent immune response by favoring their own polarization and inhibiting polarization of others T_H cell subgroups. Consequently, T_H cell polarization is continuously changing state that reflects the current internal and external needs. It is essential to restore the general balance in order to prevent T_H cell deregulation and maintain general homeostasis (Zhu et al., 2010). The general characters of the discussed T_H subpopulations are summarized in Figure 1.

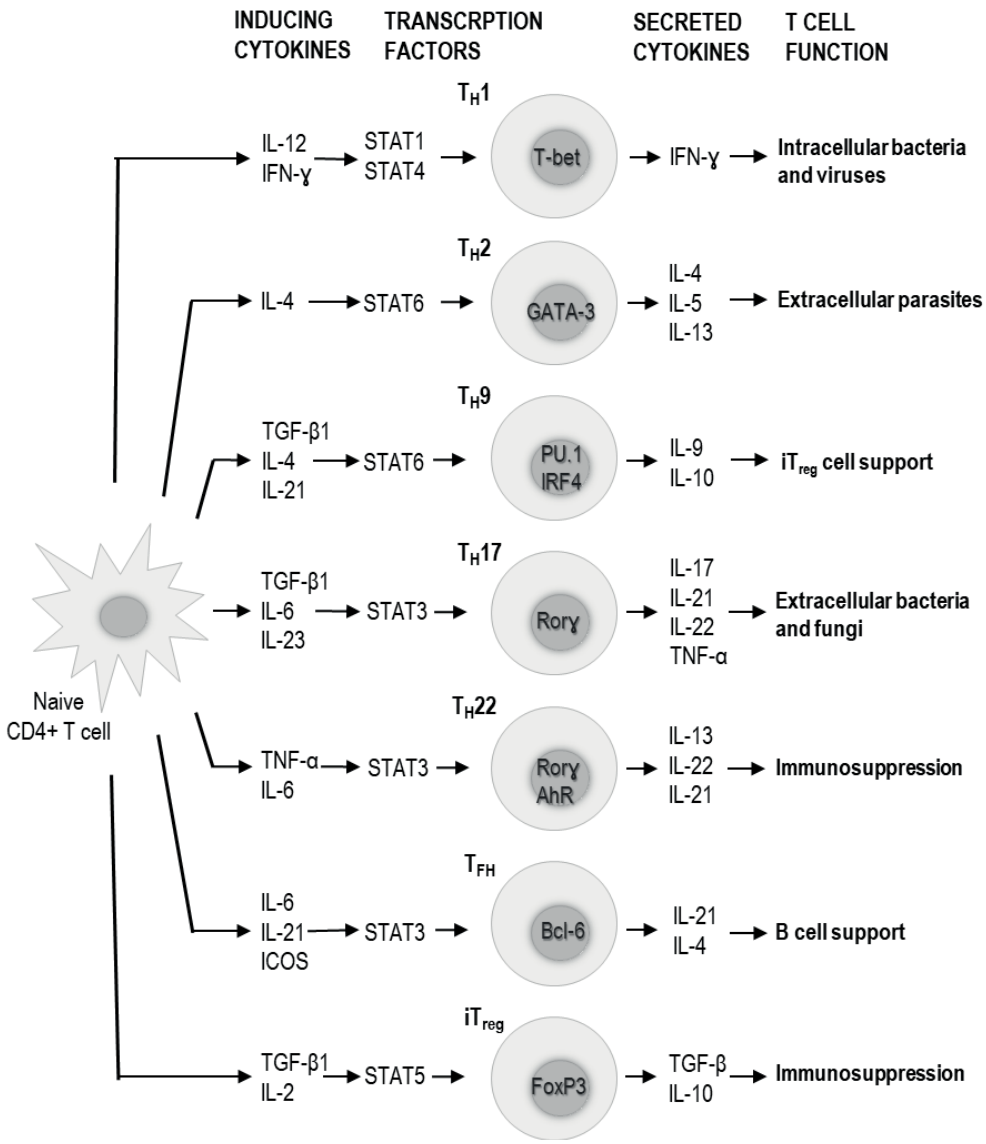

Figure 1. CD4+ T cell differentiation and function. Activation of a naive CD4+ T cell can be divided into a TCR-driven induction and a cytokine-driven polarization phase. Interaction between naive CD4+ T cell and an antigen-MHC class II complex presented by a professional APC leads to a significant increase in TCR signaling. This, together with correct second signals result in the induction of T cell activation. Present cytokine environment guides T cell polarization by activating specific STAT signaling pathways and leading to the production of a unique transcription factor repertoire. Mature CD4+ T cell subpopulations are characterized with a specific cytokine secretion that reflects their final effector functions. The figure is adapted from O´Shea, Tato, & Siegel, 2008 and Oksanen,2012.

2.2.3 T cell plasticity

Various CD4+ T cell responses are produced in response to encountered pathogens in order to ensure well-targeted actions and optimized immune responses for pathogen clearance. However, a majority of microbes are able to generate additional defense mechanisms to shelter themselves and contribute to survival. T_H1 and T_H2 cells represent relatively stable cell lines. On the contrary, studies have reported rather flexible phenotypes of T_H17 and T_{reg} cells as they are able to repolarize in response to changes in present cytokine environment. Th17 cells have been reported to turn into T_H1 cells in the presence of IL-12 or IL-23, whereas proinflammatory cytokines guided T_{reg} cells to turn into T_H1 or T_H17 cells. The ability for T cell plasticity enables relatively fast alterations in T_H-cell-mediated immune responses in order to keep up with the constantly changing demands (Murphy & Stockinger, 2010; O´Shea & Paul, 2010; Wing & Sakaguchi, 2010).

2.2.4 B cell biology

B cells originate from the same CLPs with T cells but instead of migrating to the thymus, they are kept in bone marrow for further maturation. Surrounding signaling promotes B cell gene encoding and induces the generation of characteristic BCRs. Because of the random rearrangement of BCR units, the final structure of receptor locus can vary extensively. Hence, the accurate B cell functions need to be tested carefully. Similar to T cells, general functionality is tested first via positive selection, and this is followed by autoreactivity tests via negative selection. In case of failure in central immunological tolerance tests, the cells can be returned to receptor editing. However, in case of multiple failures, they are driven to clonal deletion. Eventually, all B cells with accurate receptor functions can be released to the circulation (Kondo et al., 2001).

Newly produced naive B cells cannot live long in the bloodstream, which is why they are swiftly transported into secondary lymphatic organs where they reside in distinctive B cell zones. Lymphatic follicular cells express B cell activation factor (BAFF), which is critical for B cell differentiation, proliferation, and survival (Mackay & Browning, 2002; Pieper et al., 2013; Schiemann et al., 2001). The inflow of antigens contributes to the antigen–BCR attachment, which leads to the activation of B cells and production of soluble antibodies also known as immunoglobulins. Furthermore, activated B cells are shed to the circulation, and they migrate to infection sites where

they produce proinflammatory cytokines to enforce the local immune response (Pieper et al., 2013).

Notably, individual BCR signaling is only able to stimulate the production of IgM class antibodies, which are characterized by a relatively fast expansion but can only express low-affinity binding sites and limited effector functions. The second signaling from activated TFH cells is essential for further enhancement of antibodies (Breitfeld et al., 2000; Reinhardt, Liang, & Locksley, 2009). Moreover, TFH cells regulate the differentiation of B cells and allow the production of long-lasting memory B cells. The B cell–TFH cell interaction is, hence, pivotal for the persistent immunity and the ability for faster antibody-mediated responses in terms of reinfection (Bevan, 2011; Murphy & Weaver, 2017; Pulendran & Ahmed, 2011).

As discussed earlier, TH cells produce cytokines that guide Ig class switching and somatic hypermutation to improve both the affinity and functional capacity of produced antibodies. As a consequence, they are able to reflect the pathogen type and can provide targeted functions for a more effective clearance. Human Ig classes include IgG, IgM, IgA, IgD, and IgE antibodies; all of them differ from each other in terms of their effector functions and operation sites (Papavasiliou & Schatz, 2002; Schoenberger et al., 1998; Stavnezer, 1996).). In general terms, they can be divided into neutralizing, opsonizing, and complement-activating antibodies. In further detail, neutralizing antibodies block pathogen invasion and protect cells from intracellular infections, whereas opsonizing antibodies are able to mark pathogens for phagocytosis. Complement-activating antibodies are, in turn, specialized to facilitate complement functions through classical pathway activation (Murphy & Weaver, 2017).

2.2.5 Immunological tolerance

Because of potentially destructive acts of adaptive immune cells, the accurate identification of harmless and harmful operators is essential for survival. Pathogens that contain structures resembling host tissues create a particular challenge for recognition, as immune cells must be able to detect and eliminate them sufficiently but able to simultaneously avoid abundant response against self-structures. This state of balance in ignoring self is generally referred to as immunological tolerance. It is classically divided into central and peripheral tolerance; both contain several mechanisms that together ensure correct adaptive immune cell activation (Murphy & Weaver, 2017).

Mechanisms of central tolerance take place at the beginning of the development of lymphocytes. They consist of lymphocyte receptor testing and are divided into two stages called positive and negative selection. Positive selection confirms the general receptor functionality, whereas negative selection is important for detecting unpleasantly reacting immune cells. More specifically, tissue-specific antigens are displayed to newly formed lymphocytes during negative selection in order to edit or delete strongly autoreactive immune cells. Because of the ability to express the *AIRE* gene, thymic epithelial cells are able to create and present multiple self-antigens to pre-T cells. All highly activated lymphocytes are driven straight into apoptosis, whereas cells expressing affinity just less than the elimination limit are saved as potentially autoreactive cells and continue their development to nT_{reg} cells (Abramson et al., 2010; Von Boehmer & Melchers, 2010).

Because of the limited capacity of the *AIRE* gene, all tissue types cannot be present during negative selection. As a result, some autoreactive lymphocytes are still able to escape the central mechanisms and released to the circulation. In addition, various triggers such as environmental stress or cell aging can affect lymphocyte signaling pathways and lead to deregulated immune cell activation. Therefore, the checkpoints of peripheral tolerance are crucial in completing the central mechanisms. The most primitive method of peripheral tolerance is to simply keep adaptive immune cells away from self-tissue antigens. Naive B and T cells lodge in their characteristic zones in secondary lymphoid tissues where anti-inflammatory conditions are maintained by the presented T_{reg} cells. The continuous inflow of innate immune cells provides low-grade TCR and BCR signaling but lacks mandatory costimulatory messages. (Eberl, 2016; Murphy & Weaver, 2017). Moreover, because PRR-mediated intracellular signaling is needed for the expression of cell-surface costimulatory CD80 and CD86 receptors in APCs, the perception of individual TLR signaling indicates that the antigen is not presented by activated professional APCs. In contrast, it reflects that adaptive immune response activation is currently not needed. This activates another intracellular signaling pathway, which leads to the production of the anti-inflammatory cytokine IL-10 and drives lymphocytes to a functionally inactive state called anergy (Eberl, 2016; Macián, Im, García-Cózar, & Rao., 2004).

On the contrary, accurate lymphocyte activation stimulates adaptive immune cells to migrate into peripheral tissues wherein they are exposed to various self-antigens. Activated immune cells tend to enforce the functions of each other by creating a nonresolving cycle if not restricted. Hence, T_{reg} cells are critical in regulating activated immune cells in the periphery. They limit inflammation and prevent

excessive damage by arresting lymphocyte actions in conditions where there are no signs of danger (Josefowicz et al., 2012). The suppression of potentially autoreactive cells strengthens the eventual immune response by establishing the maintenance of sufficient detection capacity against pathogens resembling host cells.

In addition to extrinsic suppressive messages from T_{reg} cells, all immune cells are encoded with intrinsic self-limiting mechanisms to avoid excessive and prolonged inflammation. These signaling pathways are induced parallel to activation signals and lead to self-driven apoptosis after a certain period of time. Further, cells presenting exceptionally strong activation messages are promptly guided into apoptosis (Eberl, 2016).

In conclusion, immunological tolerance includes several checkpoints that assure the correct operation of the immune system. Cells expressing misleading functions are rapidly eliminated to prevent any harmful actions. Adaptive immune cells provide distinct effector functions that provide a conclusive clearance of various invasive pathogens. Immunological tolerance enables the maintenance of representative receptor collection and has a key regulatory role as an inflammation suppressor (Eberl, 2016; Murphy & Weaver, 2017). However, the breakdown of immunological tolerance allows immune cells to mount full-force responses against host cells and results in a significant self-tissue damage and unpredictable outcomes. An uncontrolled function of the immune system can lead to the generation of IMDs (DuPage & Bluestone, 2016).

2.3 Immune-mediated diseases

The failure of immunoregulation can lead to can lead to a breakdown in peripheral tolerance and cause various pathogenic conditions referred to as immune-mediated diseases (IMDs). These include, for instance, immunodeficiency, hypersensitivity, excessive inflammation, and autoreactivity. Fundamentally, redundant self-tissue damage caused by deregulated immune cells can be described as the connecting factor in all stated disorders (Murphy & Weaver, 2017). In this chapter, the pathogenesis and clinical features of three distinct IMDs are discussed in detail, including sepsis, pSS and RA.

2.3.1 Infectious diseases and development of sepsis

Sepsis is a life-threatening condition characterized by a dysregulated host response to an infection and subsequent generation of multiple organ dysfunction (MOF; Singer et al., 2016). It is a relatively common syndrome with a notably high mortality rate. A systematic review of published population-level assessments from 1979 to 2015 extrapolated a present worldwide estimate of 30 million cases of sepsis and 6 million deaths caused by it per year. Hospital mortality ranged from 17% to 26%, depending on the extensiveness of the affected organs (Fleischmann et al., 2016). Moreover, the survivors tend to suffer from long-term complications including impaired cognition, functional disability, and increased dependence. The lost productivity and increased need for health care services represent roughly 70% of the total patient costs, putting an exceptional economic burden on society (Tiru et al., 2015). In fact, the World Health Assembly and World Health Organization made sepsis a global health priority in 2017, calling states to pay further attention to improve the prevention and treatment of sepsis (Reinhart et al., 2017).

The sepsis syndrome is a rapidly progressive, overwhelming systemic inflammatory response against any infectious pathogen, leading to disadvantageous changes in microcirculation and endothelial functions (Aird, 2003; De Backer, Ortiz & Salgado, 2010; Koh et al., 2010). The loss of suppressor effects and excessive production of proinflammatory cytokines create a vicious cycle, increasingly activating stimulatory immune system components, and result in exceptionally strong oxidative stress, vasodilatation, coagulopathy, and loss of endothelial barrier functions. This leads to hemodynamic instability, impaired mitochondrial function and anergy, together with extensive tissue damage and tissue edema; all of them together contribute to organ dysfunctions (Aird, 2003; Garcia-Alvarez, Marik & Bellomo, 2014; Levi, 2001; Singer, 2014). Common manifestations of organ dysfunctiona are, for example, acute respiratory distress syndrome, acute kidney injury, and encephalopathy (Matthay, Ware, & Zimmerman, 2012; Poston & Koyner, 2019; Ziaja, 2013). Remarkably, within a few days, hyperinflammation is followed by a state of immunosuppression, making the body vulnerable to a potentially fatal second infection (Drewry et al., 2014;Otto et al., 2011; Walton et al., 2014). CD4+ and CD8+ cells are eliminated via apoptosis, and studies of spleens of patients who have died from sepsis show reduced production of cytokines and a significant decrease in T cell levels (Boomer et al., 2011; Hotchkiss et al., 2001).

Despite multiple studies, the underlying reason for the loss of immune system control remains unclear. However, the most significant risk factors for sepsis

represent conditions with general susceptibility to infections, such as very young or old age, immunosuppressive diseases, cancer, diabetes, alcohol abuse, immunosuppressive medication, parenteral nutrition, and indwelling catheters. Furthermore, genetic factors, comorbidities, and certain features of the invasive pathogen are suggested to play a role in the generation of the syndrome. Clinical manifestations of sepsis vary considerably and depend mainly on the initial infection site. Most patients arrive in the emergency room (ER) suffering from nonspecific malaise and exhibit typical symptoms of suspected infection, including fever, tachycardia, tachypnea, and altered cognition (Cecconi, Evans, Levy, & Rhodes, 2018). Comprehensive clinical examination and laboratory tests including lactate levels, white blood cell count, CRP, and procalcitonin are used to guide clinical decision-making. Moreover, because there is no specific biomarker for the sepsis syndrome, several classification criteria have been generated to facilitate the identification of sepsis from benign infections (Rhodes et al., 2017).

The traditional classification criteria for sepsis were created by the American College of Chest Physicians/Society of Critical Care Medicine (ACCP/SCCM) in 1991, focusing on recognizing general inflammation signs. The classification criteria defined sepsis as a condition with symptoms fulfilling at least two criteria for systemic inflammatory response syndrome (SIRS) and a positive blood culture sample. The term "severe sepsis" was used when sepsis was complicated by MOF and the term "septic shock" described conditions of severe sepsis and a significantly decreased mean arterial pressure (MAP; Bone, Sibbald & Sprung, 1992). The classification criteria for SIRS are described in detail in Table 1. However, further studies showed poor sensitivity and specificity for SIRS in sepsis diagnostics and prompted the development of better standards (Levy et al., 2003). The Society of Critical Care Medicine and the European Society of Intensive Care Medicine announced the Third International Consensus Definition for Sepsis and Septic Shock (Sepsis-3) guidelines in 2016 (Singer et al., 2016). The terms "SIRS" and "severe sepsis" were eliminated, and the main focus was shifted from inflammation to organ dysfunctions, using the Sequential Organ Failure Assessment (SOFA) score described in Table 2 (Vincent et al., 1996).The new Sepsis-3 guidelines define sepsis as an acute infection presenting two or more SOFA scores. Henceforth, septic shock is used when sepsis is combined with lactatemia of more than 2 mmol/L together with the need of vasopressors in order to maintain MAP greater than 65 mm Hg to avoid critical hypoperfusion (Singer et al., 2016).

Table 1. The 1992 ACCP/SCCM Consensus Committee classification criteria for SIRS.[a]

Two or more of the following criteria:

- ⇒ Body temperature >38°C or <36°C
- ⇒ Heart rate >90 beats per minute
- ⇒ Respiratory rate >20 breaths per minute or PaCO2 <32 mm Hg (4.3 kPa)
- ⇒ White blood cell count >12,000/mm3 or <4000/mm3 or >10% immature bands

[a] Adapted from the ACCP/SCCM Consensus Committee classification criteria (Bone et al., 1992).ACCP/SCCM = American College of Chest Physicians/Society of Critical Care Medicine; SIRS = systemic inflammatory response syndrome.

Table 2. The sepsis-related Sequential Organ Failure Assessment score

	Score				
System	**0**	**1**	**2**	**3**	**4**
Respiration					
$PaO2/FIO_2$ mm Hg (kPa)	≥400 (53.3)	<400 (53.3)	<300 (40)	<200 (26.7) with respiratory support	<100 (13.3) with respiratory support
Coagulation					
Platelets, $\times 10^3/\mu L$	≥150	<150	<100	<50	<20
Liver					
Bilirubin, mg/dL (μmol/L)	<1.2 (20)	1.2–1.9 (20–32)	2.0–5.9 (33–101)	6.0–11.9 (102–204)	>12.0 (204)
Cardiovascular					
	MAP ≥70 mmHg	MAP <70 mmHg	Dopamine <5 or dobutamine (any dose)[a]	Dopamine 5.1–15 or epinephrine ≤0.1 or norepinephrine ≤0.1[a]	Dopamine >15 or epinephrine >0.1 or norepinephrine >0.1[a]
Central nervous system					
GCS score[b]	15	13–14	10–12	6–9	<6
Renal					
Creatinine, mg/dL (μmol/L)	<1.2 (110)	1.2–1.9 (110–170)	2.0–3.4 (171–299)	3.5-4.9 (300–440)	>5.0 (440)
Urine output, ml/d				<500	<200

Adapted from the European Society of Intensive Care Medicine criteria (Vincent et al., 1996).
[a] Catecholamine doses are given as μg/kg/min for at least 1 hour.
[b] Scores range from 3 to 15; higher score indicates better neurological function.
FIO_2 = fraction of inspired oxygen; GCS = Glasgow Coma Scale; MAP = mean arterial pressure.

The international guidelines for the management of severe sepsis and septic shock define sepsis as a medical emergency, stating a need for urgent treatment. The most important aspects of improving patient prognosis include early detection, source control by removing infected tissues, and rapid initiation of appropriate intravenous antibiotic administration. Moreover, fluid resuscitation, vasopressors and inotropes, red blood cell transfusions, and glycemic control are used for general stabilization, but there is no evidence of their effects on the final outcomes (Rhodes et al., 2017). The mortality of sepsis is estimated to rise nearly 8% per hour of delay in the initiation of appropriate antibiotic treatment, making effective patient screening crucial (Anand Kumar et al., 2006). In parallel with the new Sepsis-3 guidelines, experts developed a new clinical sepsis screening tool, the quick SOFA (qSOFA), to facilitate early detection and treatment administration. The criteria for positive qSOFA are provided in Table 3. It is designed for bedside use to indicate an increased risk of generating sepsis and is hence suitable for use also outside the intensive care unit. The fulfillment of two or more of the following criteria indicates a positive qSOFA score: alteration in mental status, systolic blood pressure ≤100 mm Hg, or respiratory rate ≥22/min (Churpek et al., 2017). Although the qSOFA has improved the stratification of patients, it provides only indicative information of the presented condition and cannot be used for the exclusion of the sepsis syndrome. There is an urgent need for a specific early phase biomarker for the sepsis syndrome to allow for a more accurate detection of patients and possibly even a selection of targeted treatment strategies to match the individual pathophysiological profile.

Table 3. The qSOFA Score.

Two or more of the following criteria:
⇒ Altered mentation
⇒ Respiratory rate ≥22 breaths per minute
⇒ Systolic blood pressure ≤100 mm Hg

Adapted from the Third International Consensus Definition for Sepsis and Septic Shock (Sepsis-3; Singer et al., 2016).
qSOFA = quick Sequential Organ Failure Assessment.

2.3.2 Primary Sjögren's syndrome

Sjögren's syndrome (SS) is a systemic autoimmune disease characterized by irreversible exocrine gland destruction because of excessive lymphocyte infiltration. Salivary and lacrimal glands are mainly affected, leading to problematic symptoms of dry eye (keratoconjunctivitis sicca) and dry mouth (xerostomia). These are present in more than 95% of cases and are considered the hallmarks for SS (Ramos-Casals , Brito-Zerón, Sisó-Almirall, & Bosch, 2012; Vitali *et al.*, 2002). In addition, SS may affect extraglandular organs involving for example skin, joints, lungs, heart, kidneys, and nervous system, and is associated with an increased risk of B cell lymphoma. Moreover, patients with SS tend to suffer from a set of nonspecific symptoms such as fatigue, anxiety, depression, and chronic pain. In summary, the clinical presentation of SS vary widely (Mavragani & Moutsopoulos, 2014; Nocturne & Mariette, 2013; Pillemer et al., 2009).

SS is classically divided into two diagnostic groups on the basis of possible underlying diseases. Primary Sjögren's syndrome (pSS) is a disease without other autoimmune comorbidities, whereas secondary Sjögren's syndrome (sSS) is a condition where typical symptoms of SS develop after the diagnosis of an associated disease, such as systemic lupus erythematosus (SLE), RA, or systemic sclerosis (Ramos-Casals et al., 2012). The estimated prevalence of pSS varies between 0.1% and 4.8% depending on the diagnostic criteria used (Pillemer et al., 2001). It is nine times more common in females and has an incidence peak at the age of 40 to 50 years (Mavragani & Moutsopoulos, 2010; Patel & Shahane, 2014).

Despite extensive studies, the principal etiology of pSS remains elusive. It is currently suggested that an environmental factor such as a viral infection or deposition of immune complexes (ICs) might trigger undesirable lymphocyte functions in genetically susceptible individuals (Nocturne & Mariette, 2013). Genetic polymorphisms including STAT4, IFN regulatory factor 5 (IRF-5) and IL-12 subunit regulatory factor are associated with the development of pSS (Gestermann et al., 2010; Lessard et al., 2014). According to current knowledge, the main pathophysiological mechanisms are mediated through IFN-I and IFN-II pathways (Nocturne & Mariette, 2013; Pollard, Cauvi, Toomey, Morrid, & Kong, 2013). Both signaling systems promote BAFF expression and hence maintain the continuous B cell activation. This leads to the continuous production of the autoantibodies anti-Ro/Sjögren's syndrome antigen A (anti-Ro/SSA) and anti-La/Sjögren's syndrome antigen B (anti-La/SSB) distinctive for SS (Mariette et al., 2003; Vitali et al., 2002). BAFF maintains the production of IFN-α and forms a vicious circle that preserves

the pathological process (Nocturne & Mariette, 2013). Despite multiple studies, little is known about the fundamental reasons that lead to IFN-I system activation. However, at least to some extent, the elevated IFN-α expression has appeared to occur via human TLRs (Mavragani & Crow, 2010). The IFN-II pathway has been less studied even though the T_H1 cytokine IFN-γ appears to be central in the pathogenesis (Pollard et al., 2013). The prevalence of IFN-γ is necessary for the evolution of gland dysfunction in Ro/SSA immunized mice (Nocturne & Mariette, 2013). On the contrary, a decreased IFN-γ or IFN-γR level seems to inhibit the development of pSS (Pollard et al., 2013). IFN-γ expression is predominantly mediated through the IL-12/STAT4 pathway, which additionally seems to associate with an increased production of IFN-α, suggesting significant effects of IL-12 and STAT4 in the pathogenesis of pSS (Nocturne & Mariette, 2013). The ultimate failure of peripheral tolerance in pSS is caused by the decreased activity of anti-inflammatory TGF-β1, which leads to abnormal T cell polarization, impaired T_{reg} cell function, and increased production of T_H1- and T_H17-cell-type cytokines (Pesu et al., 2008).

Because of an incomplete understanding of disease-mediated events in immune regulation, there is no specific and sensitive biomarker for pSS. In addition, the current estimating manners for disease activity are inaccurate and do not reflect changes effectively. The multitier sets of criteria are at present used to assist diagnosis. The criteria comprise typical symptoms together with the presence of characteristic autoantibodies Ro/SSA and/or La/SSB or, alternatively, the presence of lymphocytic sialoadenitis in minor salivary gland biopsy. It is worth noting that invasive diagnostic examinations, such as biopsies, should be avoided if possible (Vitali, 2002). The currently used classification criteria for pSS are presented in detail in Table 4. After diagnosis, the disease activity is estimated using questionnaire-based indexes; European League Against Rheumatism (EULAR) pSS patient-reported index (ESSPRI) and EULAR pSS disease activity index (ESSDAI) (Seror et al., 2010). The complexity and nonspecificity of the used classification criteria lead to a delayed, or even missed, detection of patients with SS especially in the early phase of the disease. In addition, the currently used activity indexes react slowly and are inaccurate for the estimation of treatment outcomes. Hence, biological drugs cannot be directed to patients who benefit from them. As a matter of fact, the disease often appears to be resistant to antirheumatic drugs, and the treatment of pSS is still mostly symptomatic. The limited knowledge of the pathogenesis complicates the generation of new effective therapies (Ramos-Casals et al., 2012).

Table 4. The 2002 American–European classification criteria for pSS.

Criteria needed for the classification of a patient as having definite pSS:

The fulfillment of at least one of the following definitions together with the absence of potentially associated diseases (e.g. SLE, RA, type I diabetes, or multiple sclerosis) and any of the exclusion criteria:

(a) The presence of any four of the six items as long as either item IV or VI is positive

(b) The presence of any three of the 4 four objective criteria items (III, IV, V or VI)

Exclusion criteria:

(a) Past head and neck radiation treatment

(b) Hepatitis C infection

(c) Acquired immunodeficiency syndrome

(d) Pre-existing lymphoma

(e) Sarcoidosis

(f) Graft versus host disease

(g) Use of anticholinergic drugs (since a time shorter than fourfold the half-life of the drug)

I. Ocular symptoms: A positive response to at least one of the following questions:

(a) Have you had daily, persistent, troublesome dry eyes for more than 3 months?

(b) Do you have a recurrent sensation of sand or gravel in the eyes?

(c) Do you use tear substitutes more than three times a day?

II. Oral symptoms: A positive response to at least one of the following questions:

(a) Have you had a daily feeling of dry mouth for more than 3 months?

(b) Have you had recurrently or persistently swollen salivary glands as an adult?

(c) Do you frequently drink liquids to aid in swallowing dry food?

III. Ocular signs: Objective evidence of ocular involvement defined as a positive result for at least one of the following two tests:

(a) Rose Bengal Score or other ocular dye score (>4 in van Bijsterveld's scoring system)

(b) Schirmer's I test, performed without anesthesia (<5 mm in 5 min)

IV. Histopathology:

Focal lymphocytic sialoadenitis in minor salivary glands (obtained through normal-appearing mucosa) detected by an expert histopathologist, with a focus score ≥1, defined as a number of lymphocytic foci (which are adjacent to normal-appearing mucous acini and contain more than 50 lymphocytes) per 4 mm^2 of glandular tissue

V. Salivary gland involvement: Objective evidence of salivary gland involvement defined by a positive result for at least one of the following diagnostic tests:
(a) Unstimulated whole salivary flow (<1.5 ml in 15 min)
(b) Parotid sialography showing the presence of diffuse sialectasias (punctate, cavitary, or destructive pattern), without evidence of obstruction in the major ducts
(c) Salivary scintigraphy showing delayed uptake, reduced concentration, and/or delayed excretion of tracer
VI. Autoantibodies: The presence of autoantibodies in the serum:
(a) Antibodies to Ro/SSA antigens
(b) Antibodies to La/SSB antigens
(c) Antibodies to Ro/SSA and La/SSB antigens

Adapted from American-European Consensus Group criteria (Vitali, 2002).
pSS = primary Sjögren´s syndrome; SLE = systemic lupus erythematosus; RA = rheumatoid arthritis.

2.3.3 Rheumatoid arthritis

RA is known as one of the most common autoimmune diseases worldwide, affecting roughly 1% of the population (Silman & Pearson, 2002). It is mainly diagnosed in women, with a female-to-male ratio of 3:1(Tobón, Youinou, & Saraux, 2010). The disease is characterized by chronic inflammation of the synovial compartment, which leads to a relatively fast bone and cartilage destruction. The main symptoms of RA include joint swelling, tenderness, and stiffness, which finally results in to joint deformity. RA symptoms are most commonly present in the wrists and small joints of hands and feet (Aletaha et al., 2010) However, it can also affect extra-articular organs such as the lungs, skin, eyes, exocrine glands, skeletal muscles, and heart (Friedewald et al., 2010; Turesson et al., 2003) and is connected to an increased risk of severe comorbidities including cardiovascular diseases, osteoporosis, and hematologic malignancies (Baecklund et al., 2006; Crowson et al., 2005; Kröger, Honkainen, Saarikoski, & Alhava., 1994). Because of the broad manifestation combined with problematic diagnostics and treatment, patients with RA are estimated to lose around 3 to 12 years of life compared to healthy individuals (Friedewald et al., 2010; Gabriel, 2008).

The presence of serum autoantibodies against self-IgG-Fc (rheumatoid factor, RF) and ACPA are traditionally defined hallmarks of the disease (Aletaha et al., 2010). However, one-third of patients do not have typical autoantibodies and are identified by expressing common clinical symptoms together with increased CRP or ESR levels, indicating an abnormal acute-phase reaction (Sieghart et al., 2018).

Extensive research suggests a broad selection of etiologies and pathological pathways in seronegative RA, whereas seropositive RA appears to be much more homogenous. Seropositivity predicts more severe symptoms but associates with a better response to disease-modifying antirheumatic drug (DMARD) therapy, indicating a potential role of autoantibodies in disease development (Firestein & McInnes, 2017). Because of a better understanding of immunopathological mechanisms behind seropositive RA, its pathophysiology is mainly discussed in this chapter.

According to current knowledge, environmental factors such as long-term smoking or microbial infection might induce changes in posttranslational modification, which results in citrullination of self-proteins in mucosal surfaces (Klareskog et al., 2006; Meron et al., 2010). Consequently, autoreactive CD4+ T_H cells bind to citrulline-coated self-proteins and activate a systemic autoimmune response. The propensity to lose self-tolerance is associated with susceptibility gene polymorphism, facilitating more sensitive CD4+ T_H cell activation. HLA-DR allele that encode human leukocyte antigen (HLA) type II molecules with high-affinity binding groves is considered the main risk allele for RA (Gregersen, Silver, & Winchester, 1987). In addition, tyrosine-protein phosphatase nonreceptor type 22 (PTPN22), STAT4 and cytotoxic T lymphocyte protein 4 (CTLA4) polymorphisms have been associated with disease development (Begovich et al., 2004; Gestermann et al., 2010; Plenge et al., 2005; Remmers et al., 2007). Hypothesis of CD4+ T_H-cell-mediated pathogenesis is supported by excessive numbers of citrulline-specific T cells in the blood and accumulation of CD4+ T_H cells in inflamed joints of patients with RA (Nell et al., 2005; Smolen Aletaha, & McInnes, 2016). The production of serum autoantibodies ACPA and RF reflects T-cell-mediated activation of residing naive B cells and sequential generation of a full-force systemic autoimmune response against citrullinated self-proteins.

ICs of ACPA and RF accumulate in the synovial compartment and trigger local autoimmune responses in RA. They promote complement activation and stimulate innate immune cells to produce inflammatory mediators. Although the initiating factor that induces the transition from systemic to local disease remains unclear, vascular, neuroregulatory, microtrauma, and infection-dependent pathways are suggested to play a role in inducing tissue-specific autoimmunity in early stage RA (Firestein & McInnes, 2017). In addition to IC formation, ACPA induces a sensation of pain and promotes osteoclastogenesis directly by interacting with synovial osteoclasts (Krishnamurthy et al., 2016). The nature of signals facilitates osteoclast maturation and excessive lymphocyte infiltration. It also favors T_H1/T_H17-cell-type

polarization and leads to an increased activation of macrophages. (Firestein & McInnes, 2017). Fibroblast-like synoviocytes on the surface of the synovium induce local inflammation by assisting antigen presentation, and secrete proteases and cytokines to mediate cartilage damage (Bartok & Firestein, 2010). Remarkably, the maturation of defective T_{reg} cells is also associated with RA. Failure in peripheral tolerance enables differentiation of autoreactive lymphocytes and generation of nonresolving synovial inflammation. (Byng-Maddick & Ehrenstein, 2014)

An immunological basis of pathogenesis is supported by studies investigating immunological therapy in targeting the central pathological mediators. Patients are currently treated first with a combination of short-term glucocorticoid and general proliferation inhibitor methotrexate. However, because of incomplete treatment targeting, second line therapies are often needed and direct cytokine inhibitors together with suppressors of T and B cell activation are found to be beneficial in the treatment of RA. (Smolen, Aletaha, Koeller, Weisman, & Emery, 2007). It is worth mentioning that immunological treatments lead to disease remission in only 60% to 70% of cases. Hence, much more needs to be understood concerning the underlying reasons of tissue destruction in order to improve treatment targeting and response (Smolen et al., 2016).

Currently used biomarkers for RA, serum RF and serum ACPA, have been reported to exist several years before synovial manifestations in seropositive RA. However, these autoantibodies do not directly lead to disease generation, as they are also detected in healthy individuals (Rantapää-Dahlqvist et al., 2003). Hence, ACPA and RF tend to lack sufficient sensitivity even though they both show relatively high specificity for RA. Notable, the production of RF is also common in autoimmune diseases in general (Nell, 2005). The specificity and sensitivity rate of ACPA and RF in predicting seropositive RA are, respectively, 95% and 67% for ACPA and 85% and 69% for RF, indicating that they can only be used for strengthening the estimation and not for exclusion (Nishimura et al., 2007). However, they have been reported to show prognostic value as RF and especially ACPA positivity is associated with more aggressive disease development and support early intensive treatment.(de Brito Rocha, Baldo, & Andrade, 2019). In addition to traditional antibodies, nonspecific inflammatory markers CRP and ESR are used in the diagnosis of RA, and they have a significant role in clinical decision-making when identifying patients with seronegative RA (Aletaha et al., 2010).

In addition, new promising biomarkers for RA, such as anti-CarP and anti-PADs, were recently reported. Previous research demonstrated that serum anti-CarP antibodies have even higher specificity for RA than the traditional biomarkers and is

associated with more progressive RA. However, because of a poor sensitivity, anti-CarP could potentially only be used as a prognostic biomarker for RA (Shi et al., 2011; Spinelli et al., 2018). Likewise, determination of antibodies against PAD isoforms 2 (anti-PAD2) and 4 (anti-PAD4) together with antibodies against a subgroup of anti-PAD4 that cross-reacts with PAD3 (anti-PAD3/4), have been reported to show prognostic value in RA. More specifically, anti-PAD4 and anti-PAD3/4 were associated with more severe RA (Giles et al., 2014; Halvorsen et al., 2008) whereas anti-PAD2 was present in patients with milder symptoms and less progressive disease (Darrah et al., 2018). These findings suggest that RA have several phenotypes which differ in underlying disease pathophysiology. However, despite these promising results, questions remain and further studies are clearly needed to understand the relevance of the aforementioned antibodies in clinical field. In addition, according to current knowledge, anti-CarP and anti-PAD determinations would provide only limited advantage for RA diagnostics.

A diagnosis of RA is based on symptoms, physical examination, laboratory tests and imaging. The 2010 EULAR/American College of Rheumatology (ACR) classification criteria for RA combines multiple predicting factors and can indicate the disease. Fulfillment gives 82% sensitivity and 61% specificity meaning that the diagnosis can also be made in patients who do not meet all of the classification criteria. Therefore, the ultimate diagnosis of RA has to be made by a rheumatologist. The 2010 EULAR/ACR classification criteria for RA are presented in detail in Table 5 (Aletaha et al., 2010). The included predicting factors are typical generation of RA-specific symptoms, presence of acute-phase response, and production of autoantibodies APCA and RF. In later stages, CRP and ESR together with the development of symptoms are used to reflect the disease activity and efficacy of a given treatment.

The fact that typical symptoms are often required for the diagnosis of RA is somewhat problematic, as the symptoms generally reflect an irreversible destruction of joints. In other words, patients cannot be detected and treated in a preventive way. The future goal for clinicians is to identify patients in the pre-RA stage in order to initiate efficient DMARD therapy and decrease functional disability. New details of the pathological processes could give rise to novel inventions in the field of diagnostics and DMARD therapy.

Table 5. The 2010 EULAR/ACR classification criteria for RA.

	Score
Target population: (Who should be tested?)	
(1) Patients who have at least one joint with definite clinical synovitis (swelling)[a]	
(2) Patients with the synovitis not better explained by another disease[b]	
Classification criteria for RA (score-based algorithm: add score of categories A–D; a score of ≥6 out of 10 is needed for the classification of a patient as having definite RA[c]	
A. Joint involvement[d]:	
1 large joint[e]	0
2 - 10 large joints	1
1–3 small joints (with or without the involvement of large joints)[f]	2
4–10 small joints (with or without the involvement of large joints)	3
>10 joints (at least one small joint)[g]	5
B. Serology [h]: (At least one test result is needed for classification.)	
Negative RF and negative ACPA	0
Low-positive RF or low-positive ACPA	2
High-positive RF or high-positive ACPA	3
C. Acute-phase reactants [i]: (At least one test result is needed for classification.)	
Normal CRP and normal ESR	0
Abnormal CRP or abnormal ESR	1
D. Duration of symptoms[j]:	
<6 weeks	0
≥6 weeks	1

Adapted from the European League Against Rheumatism/American College of Rheumatology criteria (Aletaha et al., 2002).

[a] The criteria are aimed at classification of newly presenting patients. In addition, patients with erosive disease typical of RA with a history compatible with prior fulfillment of the 2010 criteria should be classified as having RA. Patients with longstanding disease, including those with inactive disease (with or without treatment) who, based on retrospectively available data, have previously fulfilled the 2010 criteria should be classified as having RA.

[b] Differential diagnoses vary among patients with different presentations, but may they include conditions such as SLE, psoriatic arthritis, and gout. An expert rheumatologist should be consulted in case of unclear relevant differential diagnoses.

[c] Although patients with a score of <6/10 are not classifiable as having RA, their status can be reassessed and the criteria might be fulfilled cumulatively over time.

[d] Joint involvement refers to any swollen or tender joint on examination, which may be confirmed by imaging evidence of synovitis. The distal interphalangeal, first carpometacarpal, and first metatarsophalangeal joints are excluded from assessment. Categories of joint distribution are classified according to the location and number of involved joints, with placement into the highest category possible on the basis of the pattern of joint involvement.

[e] "Large joints" refer to the shoulders, elbows, hips, knees, and ankles.

[f] "Small joints" refer to the metacarpophalangeal joints, proximal interphalangeal joints, second through fifth metatarsophalangeal joints, thumb interphalangeal joints, and wrists.

[g] In this category, at least one of the involved joints must be a small joint; the other joints can include any combination of large and additional small joints, as well as other joints not specifically listed elsewhere (e.g., temporomandibular, acromioclavicular, sternoclavicular).

[h] "Negative" refers to international unit (IU) values that are less than or equal to the upper limit of normal (ULN) for the laboratory and assay; "low-positive" refers to IU values that are higher than the ULN but less than or equal to three times the ULN for the laboratory and assay; "high-positive" refers to IU values that are greater than three times the ULN for the laboratory and assay. When RF information is only available as positive or negative, a positive result should be scored as low-positive for RF.

[i] Normal or abnormal is determined by local laboratory standards.

[j] The "duration of symptoms" refers to patients' self-report of the duration of signs or symptoms of synovitis (e.g., pain, swelling and tenderness) of joints that are clinically involved at the time of assessment regardless of their treatment status.

ACPA = anticitrullinated protein antibody; EULAR/ACR = European League Against Rheumatism/American College of Rheumatology; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; RA = rheumatoid arthritis; RF = rheumatoid factor

2.4 General features of proprotein convertases

Many secreted proteins, enzymes and receptors are initially synthesized as inactive precursors that need to be proteolytically processed into biologically active forms. A comprehensive reservoir of various precursors enables a fast response to alterations in internal or external demands. Proprotein convertase subtilisin/kexin (PCSK) enzymes are responsible for the proteolytic conversion of dormant promolecules and hence dictate their bioavailability. Consequently, PCSKs are important regulatory factors in biology (Seidah et al., 2008).

The PCSK protein family includes nine mammalian serine endoproteases possessing various biochemical activities. Similar to secretory proteins, PCSKs are initially produced as inactive zymogens and undergo a series of pH- and Ca2+-dependent autoproteolytic events to become enzymatically active (Seidah et al., 2008). The initially identified seven PCSKs (PCSK1–2, Furin, and PCSK4–7) originate from bacterial subtilases and yeast kexin and resemble each other in structure and biochemical features (Thomas, 2002). These conventional PCSKs are localized primarily in the secretory pathway, in endosomes, and on the cell surface. All of them cleave after C-terminal, single or paired basic amino acid lysine (K), and/or arginine (R) motif (K/R) – (X)n – (K/R) ↓, where n = 0, 2, 4, or 6 and X = any amino acid, except Cys, ↓ = the site of proteolysis (Hipp et al., 2013; Turpeinen, Ortutay, & Pesu, 2013). In vitro experiments suggest that the conventional PCSKs exhibit closely related, or even complementary, biochemical functions. However, studies with genetically engineered animal models indicate substrate specificity, which leaves fundamental relations of individual PCSKs contradictory (Thomas, 2002).

PCSK1 and PCSK2 are predominantly expressed in endocrine and neuroendocrine cells and participate in the regulation of sugar metabolisms by cleaving proinsulin and proglucagon (Day et al., 1992; Hipp et al., 2013; Roebroek et al., 1998). Overlap in the PCSK1 and PCSK2 expression patterns leads to shared substrates. However, the final outcomes can differ remarkably, as enzymes differ in proteolytic functions and cleave the same substrates in distinct manners (Turpeinen et al., 2013). PCSK4 is predominantly expressed in reproductive organs, exclusively in ovarian and testicular tissues, and its dysregulation is associated with infertility (Hipp et al., 2013; Roebroek et al., 1998). Furin and PCSK 5–6 are ubiquitously expressed and mandatory factors in embryogenesis. Hence, investigations are limited to in vivo experiments, and their fundamental cell-specific functions remain unclear (Thomas, 2002). PCSK7 is also ubiquitously expressed and has previously been

suggested to have a role in regulating iron homeostasis (Oexle et al., 2011). Notably, the last two PCSK enzymes differ greatly from the first seven conventional PCSKs, as they are highly associated with lipid metabolism. More specifically, PCSK8 has been shown to be involved in cholesterol metabolism by increasing the cholesterol and fatty acid synthesis, whereas PCSK9 specifically regulates the low-density lipoprotein (LDL) levels of serum by targeting the LDL receptors on hepatocytes for endosomal degradation (Turpeinen et al., 2011).

The changes in PCSK expression and activity are also important factors in human diseases. Some conventional PCSK enzymes, such as Furin and PCSK6, promote the malignant behavior of solid tumors (Bassi, Mahloogi, & Klein-Szanto, 2000). For example, Furin is upregulated in non-small cell lung carcinomas as well as in human head and neck squamous cell carcinomas, and the elevated Furin activity correlates positively with accelerated tumor progression (Fu, Bassi, Zhang, Li, Nicolas, & Klein-Szanto, 2015). An elevated PCSK expression also enhances the proteolytic pathogen activation. For example, genetic association studies show that a single nucleotide polymorphism on the promoter of FUR gene regulates Furin levels, which affects the replication of hepatitis B virus (Lei et al., 2009). Similarly, Furin processes exotoxin A secreted by Pseudomonas aeruginosa and consequently worsens the outcome of cystic fibrosis (Ornatowski, Poschet, Perkett, Taylor-Cousar, & Deretic, 2007).

A large genome-wide association study on blood pressure demonstrated a significant association between Furin–Fes locus and an elevated risk for cardiovascular disease (Ganesh et al., 2013). Therefore, agents that either promote or prevent PCSK function can be beneficial in the treatment of a variety of human diseases. For example, the US Food and Drug Administration approved the use of PCSK9-blocking agents (Evolocumab and Alirocumab) for the treatment of resistant hypercholesterolemia in the United States in 2015 (Chaudhary, Garg, Shah, & Sumner, 2017). Because PCSKs play a critical role in disease pathogenesis, they might have a potential value as future biomarkers in facilitating diagnostics of these diseases. Table 6 summarizes the associations between gene polymorphisms of the nine PCSK enzymes and human traits and diseases.

Table 6. Associations between PCSK gene polymorphisms and human traits and diseases.

PCSK gene	Human disease/traits association
PCSK1	Obesity Glucose homeostasis Proinsulin levels
PCSK2	Myocardial infarction Chronic kidney diseases Glucose homeostasis
FUR	Hypertension Outcome of HBV infection Aggressive tumor progression
PCSK4	Not studied
PCSK5	Neurodegenerative diseases HDL levels
PCSK6	Blood pressure Handedness in dyslexia
PCSK7	Iron homeostasis Cholesterol and lipid metabolism
PCSK8	Cholesterol and lipid metabolism
PCSK9	Cholesterol and lipid metabolism

Adapted from genome-wide association studies (Artenstein & Opal, 2011; Turpeinen et al., 2013). HBV = hepatitis B virus; HDL = high-density lipoprotein; PCSK = proprotein convertase subtilisin/kexin.

2.5 Biology of Furin (PCSK3)

Furin is the first found, and hence the most studied, mammalian proprotein convertase. It is encoded by the human C-FES/FPS proto-oncogene on chromosome 15q26.1 (Jhanwar, Neel, Hayward, & Chaganti, 1984; Roebroek et al., 1986). It is localized in the upstream region of *FES* and is, hence, also referred to as *FUR* (*FES* upstream region) gene (Roebroek et al., 1986). The novel proprotein convertase enzyme was named Furin after its encoding gene. It is expressed in all tissues and is located mostly in the trans-Golgi network in the secretory pathway. In addition, it cycles between the Golgi apparatus and the cell surface via the endosomal system (Shiryaev et al., 2007; Thomas, 2002). Furin is first produced as an immature proenzyme and has to be activated through a two-step pH- and Ca2+-dependent autoactivation. The first cleavage occurs in the neutral pH of the endoplasmic reticulum, and the second calcium-dependent cleavage is located in the more acidic environment in the Golgi apparatus. From there, it is sent to its target position (Hipp et al., 2013). Because of the wide Furin expression, its activity affects the maturation of numerous precursor proteins. Furin substrates include, for example, growth factors and their receptors, enzymes, hormones, cytokines, serum proteins, neutrophil factors, extracellular matrix proteins, and infectious agents (Thomas, 2002; Turpeinen et al., 2013). Hence, Furin is important in regulating general homeostasis.

Previous studies suggest that Furin is also involved in the pathogenesis of several diseases. For example, elevated Furin levels have been associated with aggressive cancers and metastasis (Fu et al., 2015). In addition, studies indicate that it is a significant factor in the initiation of a microbial infection. It promotes pathogen invasion by cleaving viruses' membrane proteins and the inhibiting sequences of bacterial protoxins (Shiryaev et al., 2007).

2.5.1 Furin in regulating the immune system

Previous studies have demonstrated the key regulatory role of Furin in both adaptive and innate immune responses. It is predominantly expressed in T_H1 cells and induced via the IL-12/STAT4 pathway and TCR-mediated signaling (Pesu, Muul, Kanno, & O'Shea, 2006). Moreover, the genome-wide expression quantitative trait loci (eQTL) analysis of Furin expression in peripheral blood (PB) demonstrated that,

besides extracellular signaling, Furin is also regulated genetically (Turpeinen et al., 2015).

Macrophage and T cell activation promotes Furin upregulation during an acute infection (Hipp et al., 2013; Turpeinen et al., 2011). In addition, LPS stimulates Furin expression in macrophages. Upregulated Furin promotes immune response by enhancing TLR7 and TLR9 receptor processing and stimulating IFN-γ secretion in activated T_H1 cells (Hipp et al., 2013; Pesu et al., 2006; Turpeinen et al., 2011). An elevated Furin levels has been reported, for example, in serum of patients with chronic Salmonella typhi–infection Furin has also been shown to be upregulated in lymphocytes and macrophages isolated from atherosclerotic plaques, indicating its potential role in the maintenance of chronic inflammation (Turpeinen et al., 2011). On the contrary, it has anti-inflammatory effects in LysM+ myeloid cells (macrophages and granulocytes) where it restrains the production of proinflammatory cytokines (Cordova et al., 2016). As a conclusion, relatively little is known about the fundamental role of Furin in human infections.

T-cell-expressed Furin also has an essential role in maintaining peripheral tolerance. It regulates T_H1/T_H2-cell-type polarization and functional maturity of the anti-inflammatory cytokine TGF-β1 (Oksanen et al., 2014; Pesu et al., 2006, Pesu et al., 2008;). TGF-β1, in turn, is found to be essential in the development of FoxP3+ T_{reg} cells (Haribhai et al., 2016). It also regulates Furin expression via a negative feedback loop (Blanchette, Day, Dong, Laprise, & Dubois, 1997). The key role of Furin in immunological tolerance has been demonstrated in the conventional knockout animal studies where T-cell-specific Furin knockout mice develop age-related systemic autoimmunity characterized by the clonal expansion of CD4+ and CD8+ T cells, excessive production of proinflammatory cytokines, and lack of functional FoxP3+ T_{reg} cells as a result of defective pro-TGF-β1 processing (Pesu et al., 2008). Figure 2 summarizes the main actions of Furin in regulating the immune system.

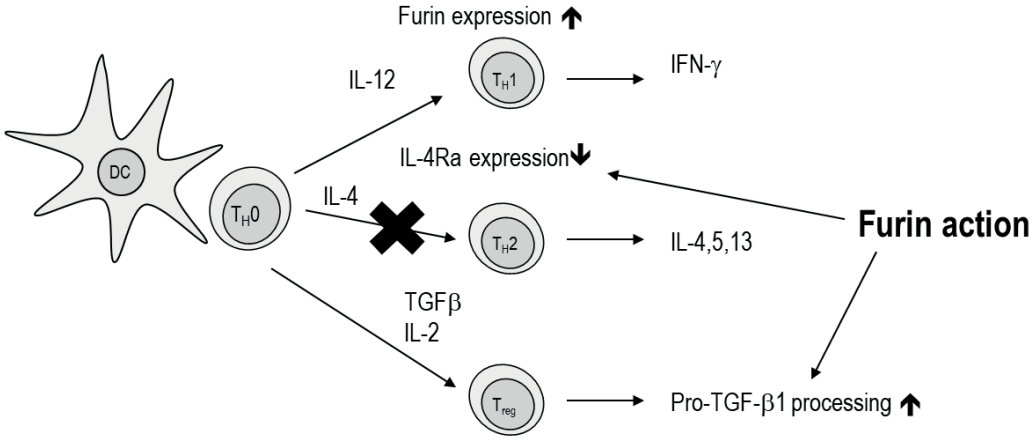

Figure 2. The role of Furin in regulating the immune system. Mature DCs migrate to secondary lymphatic organs to present antigens and activate naive T cells. Activated CD4+ T cells polarize into different subgroups on the basis of presented cytokine environment. Furin is predominantly expressed in T_H1 cells via IL-12/STAT4 mediated pathway. Furin expression in T_H1 cells inhibit T_H2 polarization by downregulating IL-4Rα expression on the cell surface of pre-T_H2 cells. Hence, Furin has an important role in the regulation of T_H1/ T_H2 balance. Moreover, T-cell-expressed Furin is also responsible for the functional maturation of pro-TGF-β1 and is therefore essential in the maintenance of peripheral immune tolerance. The figure is based on Oksanen et al., 2014; Pesu et al., 2006; and Pesu et al., 2008.

3 AIMS OF THE STUDY

Immune-mediated diseases (IMDs) are a result of a dysregulated immune system. Still little is known about the fundamental reasons for the pathogenesis of IMDs, which makes their accurate prevention, detection, and treatment a challenge. As a result, irreversible damage and lost abilities are far often present at the time of diagnosis. Hence, better tools for a timely and accurate diagnostics of IMDs are urgently needed. The PCSK enzyme Furin has a key regulatory role in both innate and adaptive immune responses. Previous studies have shown altered Furin expressions in various inflammatory conditions, including the development of metastatic cancer, infectious diseases, and atherosclerosis. However, studies to date have not yet investigated the usability of Furin measurements in clinical practice in IMDs.

The detailed objectives of the study are as follows:

(1) To investigate the clinical relevance of plasma Furin measurements in diagnostics, prognosis, and follow-up of acute infection, pSS, and RA

(2) To examine the usability of Furin messenger ribonucleic acid (mRNA) measurements in assessing clinical and immunological characteristics in pSS and RA

4 PATIENTS AND METHODS

4.1 Study population and human samples (I–III)

4.1.1 Study I—patients with suspected infection

Data for the first study was collected from the emergency department of Satakunta Central Hospital in Finland between the years 2004 and 2005. Satakunta Central Hospital is a secondary care unit with 350 beds and it provides first-aid services and intensive care for around 240 000 people in Satakunta district. Primary inclusion criteria for the study were admission to the emergency room, symptoms or clinical findings indicating an infectious disease and a medical indication for blood culture samples. Noteworthy, only patients aged 18 or more were accepted in the study. Comprehensive matching between the study and general population was assessed using a pre-evaluation that comprised demographic data from 1551 control patients. The control group consisted of consecutive first-aid patients with blood culture sample who had been previously admitted to the emergency room of Satakunta Central Hospital. No significant differences in age, gender, mortality or the rate of positive blood cultures between the two groups was obtained (Uusitalo-Seppälä et al., 2013).

The collected data consisted of plasma samples, reports from structured interviews, clinical data from days of hospitalization, medical history, and a follow-up survey. In total, 609 plasma samples were collected during the 14-month study period. They were obtained simultaneously with other blood samples and collected in two 10-ml ethylenediaminetetraacetic acid (EDTA) tubes that were preserved in ice before further processing. The samples were centrifuged in 2500 g force for 10 to 15 minutes and then shifted carefully in 1- to 2-ml aliquots to CryoPure tubes (Sartsedt, Germany) and stored at –70°C. Written consent of participation was required from the patients or, alternatively, their close relatives. As a result, 55 patients had to be excluded from further investigations. Structured interviews concerning symptoms and medical history were conducted 24 to 48 hours after admission. To ensure the fulfillment of a strict timeline, only patients admitted

between Sunday 7 a.m. and Wednesday 3 p.m. were included in the study. Comprehensive clinical data were gathered during hospitalization, which included daily observations of the highest body temperature, lowest blood pressure, highest pulse, and respiratory rates from the first 7 days of treatment. In addition, the underlying diseases, potential causes of infection, primary symptoms and clinical findings, first and final diagnosis, common risk factors for sepsis and organ failures (heart, circulation, liver, kidneys, lungs, and brain), case fatality, and sepsis-mediated case fatality were collected from medical records. Three-month and one-year follow-up checks were conducted by making phone calls.

Eventually, 17 plasma samples were excluded from the study because of the following reasons: 11 unclear results, 3 suggesting SIRS and MOF but no bacterial infection, 2 insufficient size, and 1 missing. Consequently, the final data consisted of 537 first-aid patients. These data have previously been used in other studies (Uusitalo-Sepplälä et al., 2011; Uusitalo-Seppälä et al., 2012; Uusitalo-Seppälä et al., 2013; Uusitalo-Seppälä, Peuravuori, Koskinen, Vahlberg, & Rintala, 2012).

The ACCP/SCCM Consensus Committee definitions were used for dividing patients into five diagnostic groups with respect to disease severity in order to assess associations between Furin plasma levels and clinical manifestations (Bone et al., 1992).

4.1.2 Study II—patients with pSS

Participants in the second study were recruited from the Centre for Rheumatic Diseases in Tampere University Hospital, Finland. In total, 16 patients with the active state of pSS were enrolled. Detailed inclusion criteria covered the fulfillment of four or more revised American–European consensus group criteria for pSS and a verification of systemic disease activity with either ESSDAI score >11 or laboratory test results: ESR >20 mm/h, IgG >15 g/L, serum β2-microglobulin >2.2 mg/L, or serum complement C4 <0.10 g/L (Vitali, 2002). The final group consisted of 14 female and 2 male patients, with a median age of 53 years (range 32–80 years), disease duration of 11 years (range 0–27 years), and disease activity index (ESSDAI) of 5.00 (interquartile range 3.00–8.75). Five patients were treated with immunosuppressive medications at the time of sampling.

PB samples were collected into BD Vacutainer tubes (BD Biosciences, San Jose, CA, USA) containing acid citrate dextrose (ACD) solution B as an anticoagulant and were stored at −70°C. Furthermore, 10 peripheral blood mononuclear cell (PBMC)

samples and 14 control plasma samples were drawn randomly from the pool of healthy blood donors (Finnish Red Cross Blood Transfusion Service, Tampere, Finland). Clinical and laboratory data of patients with pSS were gathered from the patients' records. The data have been used in previous publications (Pertovaara, Silvennoinen, & Isomäki, 2015; Pertovaara, Silvennoinen, & Isomäki, 2016).

4.1.3 Study III—patients with RA

Participants in the third study were recruited from the Centre for Rheumatic Diseases in Tampere University Hospital. The predominant criterion for inclusion was the diagnosis of an active RA. In accordance with scientific principles, written consent was obtained from every participant. The total data for the study comprised 17 plasma samples, 16 PBMC samples, 8 synovial fluid mononuclear cell (SFMC) samples, and 8 sets of purified T cells and monocytes obtained from patients with RA. Control samples were gathered from anonymous healthy blood donors (Finnish Red Cross Blood Transfusion Service).

Clinical and demographic characteristics of the patient groups were as follows: The plasma sample group consisted of 4 men and 13 women, with a median age of 66 years (range 52–88 years), median disease duration of 7 years (range 0–29 years), median CRP of 18 mg/L (range 0–68 g/L), and median ESR of 40 mm/h (range 7–118 mm/h). The PBMC sample group consisted of 7 men and 9 women, with a median age of 66 years (range 32–88 years), median disease duration of 15 years (range 0–37 years), median CRP of 30 mg/L (range 11–114 g/L), and median ESR of 42 mm/h (range 10–118 mm/h). The SFMC sample group consisted of 6 men and 2 women, with a median age of 54 years (range 32–72 years), median disease duration of 11 years (range 1–37 years), median CRP of 44 mg/L (range 15–114 g/L), and median ESR of 32 mm/h (range 10–88 mm/h). The purified T cells and monocytes group consisted of 8 patients, with a median age of 62 years (range 37–87 years), median disease duration of 18.5 years (range 0.25–49.0 years), median CRP of 25 mg/L (range 18–67 mg/L), and median ESR of 36 mm/h (range 10–91 mm/h). Previous and current use of immunosuppressive treatment was allowed in every patient group.

4.2 Measurement of Furin plasma levels (I–III)

The measurements of serum Furin levels were carried out using a commercial Human Furin ELISA (enzyme-linked immunosorbent assay) Kit according to the manufacturer's instructions in all three studies (Sigma-Aldrich, USA (I and II) or Thermo Scientific, USA (III)). In study I, samples showing exceptionally high protein levels were analyzed again using higher dilution, and for studies II and III, duplicated analyses were performed for every sample to ensure reliable results. The detection limit of ELISA varied between patient groups as follows: 370 pg/ml for first-aid patients (I) and 123 pg/ml for patients with pSS or RA (II and III). All concentrations less than this were considered to represent low (I and III) or absent (II) protein levels. For all studies, the patients were further stratified into low- and high-plasma Furin groups in accordance with detection threshold (370 pg/ml for study I and 123 pg/ml for study III) or median plasma Furin concentration (2690 pg/ml for study II).

4.3 Cell purification (II and III)

To study Furin mRNA expression in pSS in study II, PBMCs were isolated from blood samples using Ficoll-Paque Plus (Amersham Biosciences, Buckinghamshire, UK) density gradient centrifugation. Respectively, examinations of the *FUR* gene expression in patients with RA in study III followed an identical protocol, except that SFMCs were isolated from synovial fluid samples.

For study III, additional T cells and monocytes were isolated from eight patients with RA, using magnetic beds (Miltenyi Biotec, Auburn, CA, USA; III) and sequential negative selection (Pan T Cell Isolation Kit; Miltenyi Biotec) for T cells and positive selection with anti-CD14-coated microbeads for monocytes. The ribonucleic acid (RNA) from purified cells was further extracted for quantitative real-time polymerase chain reaction (Q-RT-PCR) experiments (II–III).

4.4 Q-RT-PCR (II and III)

In study II, the total RNA from human PBMCs was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions and reverse transcribed into complementary deoxyribonucleic acid (cDNA) with Maxima

Reverse Transcriptase and random hexamers (Thermo Scientific, Waltham, MA, USA). An identical protocol was followed when isolating the total RNA from human PBMCs and SFMCs for study III.

The Primer3 software (available at http://primer3.sourceforge.net) was used to design forward and reverse primers for Furin and TATA-binding protein (TBP, housekeeping) for studies II and III. The following primer sequences were used: 5'-GGCAAAGCGACGGACTAAAC-3' and 5'-CGTCCAGAATGGAGACCACA-3' for Furin, and 5'-GAATATAATCCCAAGCGGTTTG-3' and 5'-ACTTCACATCACAGCTCCCC-3' for TBP (Pertovaara et al., 2015). Q-RT-PCR analyses were performed using a CFX96 instrument together with Maxima SYBR Green/ROX (Thermo Scientific) master. TBP was used for normalization of the Furin expression in both studies (II and III) in order to minimize errors in sample quantifications. The relative Furin expression was calculated by dividing the mean expression level of Furin by the mean expression of the TBP obtained from triplicated samples.

4.5 Statistical analyses (I–III)

Data management and analysis were performed using SPSS version 22.0 (IBM Corp., Armonk, NY, USA) in studies I and III and version 20.0 in study II. Statistical significance was calculated using different analyses depending on the variable type, distribution, and sample size. In study I, Student's t-test and analysis of variance were used for normally distributed variables, and the Mann–Whitney U-test and Kruskal–Wallis test were used to evaluate the statistical significance of variables from non-Gaussian population data. Chi-squared and Fisher's exact test were used in assessing the statistical significance between binomial variables. Regression analysis was used for investigating the effects of potential confounders. The fatality in different Furin plasma levels was assessed using the log-rank (Kaplan–Meier) test.

The nonparametric Mann–Whitney U-test was used to determine the statistical differences in patients with pSS (II) and RA (III). In addition, Fisher's exact test was used in Furin mRNA expression analyses in study III in terms of analyzing sample sizes less than n = 50. Spearman's correlation coefficient was used to determine the associations between gene expression level and clinical and demographic parameters (II–III).

Common significance levels were used in all analyses: p-values < 0.05 were considered statistically significant, and p-values < 0.001 indicated a high statistical significance (I–III).

4.6 Ethical considerations (I–III)

The collection of human plasma samples and their use in studying infectious diseases were approved by the Ethics Committee of Satakunta Central Hospital (I). Correspondingly, the permission to collect blood and synovial fluid samples from patients with pSS (II) and RA (III) together with their use in examining the pathogenesis of rheumatic diseases was given by the Ethics Committee of Tampere University Hospital. New patient samples or patient data were not collected during the presented studies. All patients were comprehensively informed about the use of collected samples and their personal medical records in the following clinical trials. In every study (I–III), written consent was required from either the participants or their close relatives. Stored personal data were processed and described by research numbers to achieve anonymity and protect patients' privacy.

5 SUMMARY OF THE RESULTS

5.1 Furin plasma level as biomarker for suspected infection (I)

In study I, ELISA measurements were used as described in Section 4 to analyze the Furin plasma levels from 537 first-aid patients admitted to the ER, with clinical signs suggesting infectious diseases. In addition, medical history, demographic characteristics, and infection-associated clinical parameters for the time of hospitalization and results from two follow-up surveys were collected to investigate the associations between initial Furin plasma levels and disease outcomes. Follow-up surveys were conducted 3 months and 1 year after the initial admission to the ER. In addition, we assessed the utility of Furin plasma measurements in diagnosis and initial treatment selection for the early phase of infection.

In particular, Furin ELISA measurements turned out to be technically problematic. Concentrations less than 370 pg/ml could not be reliably detected, and hence, the patients were further classified into high- and low-level Furin groups, with a cutoff level at the detection threshold. Further, the patients were divided into five diagnostic groups according to their clinical findings and symptom severity on the basis of the ACCP/SCCM Consensus Committee definitions: group 1, no SIRS or bacterial infection (n = 59 patients); group 2, bacterial infection without SIRS (n = 67); group 3, SIRS without bacterial infection (n = 308); group 4, sepsis without organ failure (n = 308); and group 5, sepsis and organ failure (n = 49; The presence of high Furin plasma levels varied from 14.9% to 22.0% among these five diagnostic groups. Low Furin plasma levels were concentrated in group 2, whereas group 1 showed most predominantly high Furin plasma levels, suggesting that Furin might have a role in preventing pathogen invasion. However, no significant correlation was found among the different groups and the Furin plasma levels ($p = 0.737$).

Next, we investigated if Furin plasma measurements could be beneficial in predicting changes in general infection-associated clinical parameters by assessing their correlations with the groups of patients with high- and low-level Furin. We also studied the connections between Furin plasma level and underlying diseases, as well as demographic characteristics, to discover potential risk factors for distinct Furin expression. Despite the fact that an increased Furin expression has been

demonstrated in atherosclerotic plaques and malignancies (Li et al., 2010; Thomas, 2002; Turpeinen et al., 2011), no evidence of correlation between the prevalence of solid cancer ($p = 0.606$) or cardiovascular diseases ($p = 0.204$) was found in patient groups. However, consistent with earlier studies demonstrating Furin upregulation in the synovial pannus of patients with RA (Lin et al., 2012), an elevated Furin plasma level was observed to correlate strongly with the presence of rheumatic diseases ($p < 0.001$). Interestingly, in contrast to 7.0% representation of rheumatic diseases in the low-level Furin group, even 18.5% of patients expressing high Furin plasma levels had a previous diagnosis of rheumatic diseases. On the contrary, 40% of patients with underlying RA presented high Furin plasma compared to 18% in the patient group with no history of rheumatic diseases. We also noted a surprising connection between current smoking and a tendency for expressing low Furin plasma levels ($p = 0.034$). Taken together, these results suggest that Furin plasma levels at least when determined with the used ELISA measurements cannot be used as a diagnostic biomarker for first-aid patients with suspected infection.

The next section of study I evaluated the potential use of Furin as a prognostic marker for ER patients with infectious manifestations. However, the analysis showed no significant differences between Furin plasma levels and development of sepsis ($p = 0.957$), multiorgan failure ($p = 0.421$), or any of the other clinical manifestations during the first 28 days of hospitalization. Furin expression was not found to be associated with 28-day case fatality among patients who died or developed severe illness as a result of the sepsis syndrome ($p = 0.463$). Next, two survival curves were constructed to further investigate the predicting value of Furin plasma levels in disease severity. Because Furin is unregulated in activated T cells (Pesu et al., 2006) and dysregulation of T cell response appears to play a pivotal role in developing sepsis (Yang, 2014), we targeted our survival analysis to patients who were initially diagnosed with sepsis (groups 4 and 5). Because sepsis-related deaths tend to occur during the first month, the analysis was focused on the first 28 days of treatment. The presence of any other direct causes of deaths during the determined time limit resulted in exclusion from the analysis ($n = 13$). However, no evidence of significant association between the initial Furin plasma level and the case fatality of patients with sepsis was found ($p = 0.898$). The data did not show a significant difference between Furin levels and case fatality during 1-year follow-up surveys ($p = 0.742$).

Lastly, we assessed the potential value of Furin plasma measurements in the initiation of empiric antimicrobial treatment. The patients were divided into four groups in accordance with blood culture findings to analyze the potential differences

in Furin expressions. These were as follows: no growth (n = 485), gram-positive finding (n = 28), gram-negative finding (n = 16), and mixed infection (n = 7). Because LPSs have been shown to induce Furin expression in macrophages (Kumar et al., 2014; Meissner, Scheltema, Mollenkopf, & Mann et al., 2013), we expected to see differences between dissimilar microbial infections. However, no significant difference in these four groups was evident (p = 0.351). Next, we determined the distribution of Furin concentration groups between gram-positive and -negative bacterial growth and also between single bacterial species separately. Gram-negative *Escherichia coli* (n = 11) and gram-positive *Streptococcus pneumoniae* (n = 10) were the most common causes of bacteremia in our study group. In parallel to previous analyses, none of the differences concerning Furin plasma measurements and individual bacterial species were statistically significant. These results suggest that Furin plasma measurements cannot be used in decision-making of the initial antimicrobial treatment.

In summary, the used ELISA measurements of Furin plasma level cannot be used as a biomarker for first-aid patients with suspected infection. In more detail, the Furin plasma level did not predict blood culture results, disease severity, diagnostic group, or case fatality. However, because high Furin levels appeared to associate with underlying rheumatic diseases, Furin could potentially have a better value as a biomarker for autoimmune diseases.

5.2 Furin as biomarker for pSS (II)

Because study I showed an interesting correlation between Furin plasma levels and rheumatic diseases, we next assessed the potential usability of Furin measurements as a biomarker for autoimmune diseases. In study II, we focused on determining Furin expression levels in patients with pSS to evaluate whether or not they could be used to facilitate diagnostics and clinical decision-making in this study group. In addition, the associations between Furin expression levels and clinical and demographic characteristics of patients were assessed to detect any causal relationships. Because of the problematic use of ELISA in study I, we broadened our method repertoire to also cover Furin mRNA measurements from isolated PBMCs, using the Q-RT-PCR method. We also changed the manufacturer of the ELISA Kit for this study. Nonetheless, similar detection deficiency of the ELISA Kit was once again faced. The reliable detection limit of new ELISA in study II was 123 pg/ml, and all samples with a concentration less than this threshold were

considered to have the absence of the protein. The Furin plasma levels were compared between patients with pSS and healthy controls to evaluate whether or not Furin was elevated in pSS. Our results showed that Furin was significantly higher in patients with pSS compared to healthy controls (median 0 vs. 2690 pg/ml; p = 0.0181). Next, we compared the Furin mRNA expression in PBMCs between patients and controls in order to estimate if the detected elevation was caused by speeding up the protein secretion or by increase in gene expression. Furin mRNA expression in isolated PBMCs was found to be significantly higher in patients with pSS (p = 0.0061), indicating that the *FUR* gene is upregulated in patients with pSS. This finding is consistent with the earlier findings considering Furin measurements from plasma samples.

In the second section of study II, we investigated whether or not the elevation in Furin plasma levels was associated with demographic, clinical, or disease-associated immunological features of patients with pSS. The patient group was divided into a high- and low-level Furin groups because of the insensitive detection of Furin levels less than 123 pg/ml. The median Furin concentration was 2690 pg/ml, and it was used as a cutoff level for the two concentration groups. On average, patients with high Furin plasma levels were also reported to have significantly longer duration of the symptoms of dry eye than those showing low Furin concentrations (median 13 years vs. 9 years; p = 0.035). In addition, they had significantly elevated IFN-γ levels (median 100.0 pg/ml vs. 27.2 pg/ml; p = 0.036). The correlations between Furin plasma levels and IFN-γ levels (Spearman's correlation, r = 0.631; p = 0.009) and with the duration of the symptoms of dry eye (r = 0.520; p = 0.038) were confirmed to be statistically significant in subsequent analyses. No other statistically significant difference between patients' characteristics and Furin plasma concentration was detected. However, there was an interesting trend for lower serum β2-microglobulin level (median 2.30 mg/L vs. 3.05 mg/L; p = 0.050), ESR (median 11 mm/h vs. 29 mm/h; p = 0.138), and systemic disease activity index ESSDAI (median 3.00 vs. 6.50; p = 0.205) in patients with pSS having high Furin levels compared to the low-level Furin group.

When taken together, our results demonstrated that the proprotein convertase Furin is significantly upregulated in pSS at both protein and mRNA levels and there was a significant association between high Furin levels and longer duration of the symptoms of dry eye and plasma IFN-γ levels. Contradictory, high Furin levels in plasma also tend to associate with smaller scores of disease activity indicators including beta-2 microglobulin, ESR and the systemic activity index ESSDAI, although this did not reach the statistical significance level.

5.3 Furin as biomarker for RA (III)

Because both of our previous studies showed significant associations between elevated Furin levels and rheumatic diseases, suggesting its potential role in the pathogenesis, we wanted to perform further examinations concerning Furin expression in autoimmune diseases. We assessed Furin expressions in patients with RA, as it is one of the most common autoimmune diseases worldwide. We also investigated the potential utility of Furin measurements as a biomarker for RA by evaluating results' associations with the common RA-associated clinical features.

Similar ELISA measurements as in the study on pSS were used for determining the Furin plasma level from 17 patients with active RA. The reliable detection limit for ELISA was 123 pg/ml, and concentrations less than this were marked as 0 pg/ml. In contrast to our previous studies of Furin in plasma, no statistically significant increase was detected when comparing patients with RA to healthy controls ($p = 0.23$). In fact, a high variability of Furin plasma levels was demonstrated in both groups. Nevertheless, there were higher detected Furin plasma levels among patients with RA (10 out of 17, 59%) compared to healthy controls (5 out of 14, 35%).

Next, the Furin mRNA expression level in isolated PBMCs was assessed from patients with active RA and healthy controls, using the Q-RT-PCR method. Statistical analysis showed that Furin mRNA expression in purified PBMCs from patients with RA showed significantly higher levels than did the control group ($p < 0.001$). Furthermore, T cells ($n = 7$) and monocytes ($n = 6$) from the PB samples of patients with RA and healthy controls were further purified to investigate Furin mRNA expressions between distinct PBMC types. Our results demonstrated significantly higher Furin mRNA levels in PB T cells ($p < 0.05$) as well as PB monocytes ($p < 0.01$) in patients with RA compared to healthy controls. Moreover, Furin mRNA upregulation was higher in PB monocytes compared to PB T cells in examined samples from patients with RA. Because examinations of isolated PBMCs reflect mainly systemic effects, mononuclear cells were also isolated straight from synovial fluid in order to get better insights into the local alterations. Synovial fluid samples were taken from inflamed knee joints of eight patients with active RA. SF T cells ($n = 4$) and SF monocytes ($n = 4$) were purified from extracted SFMCs, using similar methods as described earlier with PBMCs. The detected Furin mRNA expression was in parallel with the analysis considering PB cells. The finding suggests a consistency in systemic and local changes in transcription of the *FUR* gene in the presence of active RA. In summary, although there was no significant difference in

Furin plasma levels between patients with RA and healthy controls, Furin was found to be highly significantly upregulated in RA at mRNA levels in both PB and SF mononuclear cells.

The next section of study III evaluated the associations between Furin levels and characteristics of patients with active RA. First, we investigated whether or not Furin plasma was related to demographic or clinical features of patients with RA. Patients having detectable Furin levels had significantly higher doses of prednisolone (p = 0.023) and a greater Health Assessment Questionnaire (HAQ) disability index (p = 0.003) compared to those with lower Furin concentrations. Moreover, additional cytokine levels (IL-1β, IL-2, IL-4, IL-6, IL-7, IL-10, IFN-γ, and TNF-α) were determined from 10 plasma samples of patients with RA to evaluate their association with Furin plasma concentrations. Our analysis showed that patients expressing detectable Furin levels (7 out of 10) also demonstrated higher median levels of all evaluated cytokines when compared to those with low Furin plasma levels (3 out of 10). However, differences concerning only IL-2 levels were found to be statistically significant (median 28 pg/ml vs. 6 pg/ml; p = 0.033).

Second, we assessed if upregulated Furin mRNA would associate with clinical or demographic characteristics of patients with RA, using a similar protocol as earlier. A significant positive correlation was found between high Furin mRNA expression in PBMCs and current use (p = 0.020) and dose (p = 0.004) of prednisolone. In turn, patients with high Furin mRNA expression in SFMCs showed significantly higher acute-phase CRP levels (p < 0.001). In addition, there was a trend toward higher ESR level (r = 0.6667; p = 0.0710) and a higher prednisolone dose (r = 0.6747; p = 0.0664) in patients with RA having higher Furin mRNA expression in SFMCs.

6 DISCUSSION

6.1 Furin in suspected infection

Infectious diseases are common problems in patients admitted to the ER. Although most of the conditions turn out to be harmless, dysfunctions in the regulation mechanisms of host defense can lead to life-threatening outcomes. The failure of suppressor functions leads to uncontrolled inflammation, which is associated with severe self-tissue damage and organ failures. Sepsis is defined as a condition with infection-related acute organ dysfunctions (Cecconi et al., 2018). It is considered a significant global health issue because it affects all age groups and is associated with a high risk of death. (Fleischmann et al., 2016). It is important to note that characteristic organ dysfunctions progress relatively fast, hence making urgent diagnosis vital. Consequently, even a short delay in the diagnosis and initiation of antimicrobial treatment is related to increased mortality (Anand Kumar et al., 2006). Conventional inflammatory biomarkers, such as CRP, do not accurately reflect the early phase immune cell activation (Clyne & Olshaker, 1999). Hence, novel biomarkers for sepsis are critically needed, with a particular reference to ones that would allow the early detection of these immune dysfunctions.

Previous studies have demonstrated that Furin is upregulated upon immune cell activation and has a key role in regulating host defense. It is readily induced upon both T cell and macrophage activation in toxoplasma- and mycobacteria-infected animals (Ojanen et al., 2015; Oksanen et al., 2014). Further human studies have additionally strengthened its connection with the pathogenesis of various inflammatory conditions (Hipp et al., 2013; Kumar et al., 2014; Meissner et al., 2013; Pesu et al., 2006, Pesu et al., 2008; Shiryaev et al., 2007; Turpeinen et al., 2011). Because a part of Furin is secreted to the extracellular space, Furin levels can be reliably assessed in fluids. Here, we determined Furin plasma levels in a group of first-aid patients with suspected infection, using a commercial ELISA Kit (R&D Systems, Sigma-Aldrich). The central aim was to evaluate the biomarker potential of Furin plasma measurements in the early phase diagnostics and clinical decision-making. The results of our study indicated that Furin is not suitable as an early phase diagnostic biomarker for suspected infection in the ER, at least when measured with

the aforementioned ELISA kits. Our Furin plasma measurements from first-aid patients with suspected infection appeared to bring no additional value in treatment planning or assessment of the final prognosis. Our results also indicated that Furin does not reflect the bacterial species, gram staining, or ultimate case fatality. However, we found that patients with underlying rheumatic diseases tend to have significantly higher Furin plasma levels when compared to healthy controls. Furthermore, Furin expression during infectious conditions appeared to have a connection with patients' smoking habits, as nonsmokers had considerably higher Furin concentrations in plasma than healthy controls generally.

Our finding concerning the elevated Furin expression in rheumatic diseases is consistent with previous studies because increased Furin expression and activity has been demonstrated in the inflamed synovial pannus in RA (Lin et al., 2012). Even though the fundamental role of Furin in the pathogenesis of autoimmunity is still unclear, Furin appears to have a key regulatory role in the maintenance of peripheral self-tolerance. It promotes the bioavailability of the anti-inflammatory TGF-β1 and regulates proper anti-inflammatory functions of CD4+ FoxP3+ T_{reg} cells (Pesu et al., 2008). Interestingly, the systemic use of recombinant Furin has been shown to suppress inflammation and prevent joint destruction in experimental collagen-induced arthritis in a mouse model (Lin et al., 2012). According to that, elevated Furin plasma levels could reflect a compensatory attempt to harness overly active host defense by anti-inflammatory response activation. However, because T-cell-expressed Furin also regulates T_H1/T_H2-cell-type polarization by favoring a T_H1-cell-type response, further studies are clearly needed to understand the immunoregulatory role of Furin in plasma (Oksanen et al., 2014).

The fact that smoking is one of the most relevant environmental risk factors for RA places our finding suggesting an association between high Furin expression and nonsmoking to an intriguing position (Klareskog et al., 2006). Because smoking is generally demonstrated to induce proinflammatory cytokine secretion and stimulate chronic inflammation, the association appears somewhat counterintuitive (Lee, Taneja, & Vassallo, 2012). Perhaps prolonged smoking leads to increased presentation of antigens in the lungs. This could cause a constant susceptibility state for the breakdown of immunological tolerance and lead to strong Furin expression during immune cell activation. Hence, Furin would not be elevated as excessively among nonsmokers during an acute infection. The other explanation could be that tobacco-related substances in plasma interfere with the Furin measurement, hence falsifying our data. Therefore, analyzing Furin mRNA or protein expression directly

in the lungs could provide more reliable data on the effect of smoking on Furin levels.

To our knowledge, the usability of Furin as a diagnostic biomarker for infections has not been previously assessed. Because of the comprehensive and randomly collected study group, the presented findings are well generalized to the Finnish population. Although measuring Furin in plasma samples was not valuable in clinical decision-making in this study, we could not exclude the potential usability of Furin levels lower than 370 pg/ml. More sensitive measurement techniques are needed to evaluate the importance of lower Furin plasma levels. Altogether, the presented findings call for further investigations relating to the potential use of Furin plasma measurements as a biomarker for autoimmune diseases.

6.2 Furin in pSS

pSS is a systemic autoimmune disease that mainly affects the lacrimal and salivary glands. Dysfunctions in both humoral and T-cell-mediated immune responses cause excessive activation and proliferation of autoreactive immune cells, leading to ultimate gland destruction (Nocturne & Mariette, 2013). However, because the fundamental mechanisms behind pSS are still unclear, clinicians have no specific diagnostic methods to detect incipient pSS (Vitali, 2002). This leads to delayed, or even missed, diagnosis and causes significant damage and disability. Novel biomarkers for pSS, and for autoimmunity in general, are critically needed to avoid these irreversible injuries. The proprotein convertase enzyme Furin is highly expressed in T_H1 cells and has a key regulatory role in the maintenance of peripheral tolerance (Pesu et al., 2008). It has previously been shown to associate with various diseases presenting nonresolving inflammation, such as atherosclerosis, arthritis, and SLE (Lin et al., 2012; Turpeinen et al., 2011; Wu et al., 2016). Consistent with earlier findings, our previous study considering first-aid patients with suspected infection (I) suggested that high Furin plasma levels are associated with diagnosed rheumatic diseases. These aforementioned reasons prompted us to evaluate the potential value of Furin measurements in the context of autoimmunity.

In study II, we investigated Furin expression levels in pSS to assess its usability as a clinical biomarker is this patient group. Because our previous measurements of Furin plasma levels using a commercial ELISA Kit failed to detect low protein levels, we wanted to improve our determination methods for this study. Hence, we used other manufacturer's ELISA Kit and decided to add mRNA determinations to our

assessments in order to get better insights into Furin expression. The transcripts of Furin mRNA were determined from isolated PBMCs, using the Q-RT-PCR method as described in Section 4.

In study II, we demonstrated that both high Furin plasma levels and high Furin mRNA expressions are associated with elevated plasma levels of the proinflammatory cytokine IFN-γ. This type II IFN is predominantly secreted from T lymphocytes and has been shown to coordinate various cellular acts in host defense. IFN-γ, for example, mediates Th1 cell functions, activates macrophages, and facilitates Ig class switching (Boehm, Klamp, Groot, & Howard, 2002; Carnaud et al., 1999; Finkelman, Katona, Mosmann, & Coffman, 1988; Young & Hardy, 1995). Moreover, elevated IFN-γ expression has been universally associated with the presence of systemic autoimmunity (Pollard et al., 2013). In pSS, more precisely, IFN-γ has been shown to be important in the development of glandular destruction (Nocturne & Mariette, 2013). Interestingly, both Furin mRNA and IFN-γ are expressed in T_H1 cells and regulated via the same IL-12/STAT4 pathway. Hence, T_H1 cells express these molecules simultaneously, which can partly explain this relationship (Pesu et al., 2006; Pollard et al., 2013). Nevertheless, in this study we demonstrated the association between elevated Furin and IFN-γ protein levels in PB for the very first time.

In the present study, we also demonstrated that altered Furin plasma levels associate with clinical parameters in pSS. Patients with pSS having an increased Furin plasma level appeared to suffer from a considerably longer duration of the symptoms of dry eye. However, we also noted a nonsignificant trend in which patients with pSS having high Furin plasma levels seemed to possess relatively lower rates in disease activity indicators, such as serum β2-microglobulin levels and ESR levels, as well as the systemic disease activity index ESSDAI. Previous studies have been arguing for a crucial role for Furin in the maintenance of peripheral tolerance by dictating the functional maturity of TGF-β1 (Cordova et al., 2016; Pesu et al., 2008). In addition, Furin deletion results in impaired Th cell functions because it has been shown to associate with the development of less protective $FoxP3^+$ T_{reg} cells and oversensitive effector T cells with reduced susceptibility for suppressive signals of wild-type T_{reg} cells (Pesu et al., 2008).

Because elevated Furin plasma levels were nonsignificantly associated with a trend toward milder clinical findings, our results could argue for a potential symptom-reducing function of Furin in pSS. As previously mentioned, similar results have been reported in studies investigating systemic Furin in patients with RA, where it was shown to reverse the T_H1/T_H2 balance in inflamed joints and lead to a greater

proportion of T_{reg} cells in the spleen (Lin et al., 2012). The fact that active Furin has been shown to prevent the production of proinflammatory cytokines in macrophages (Cordova et al., 2016) additionally supports its protective role in nonresolving inflammation. However, the fundamental nature of Furin in regulating the immune system appears to be multifaceted. Although an important anti-inflammatory role of Furin seems evident, it also performs an important proinflammatory function, as it promotes various proinflammatory cytokines including BAFF (Turpeinen et al., 2011). Further studies of *FUR* gene expression showed no connections with demographic, clinical, or immunological features of study patients, indicating that the assessment of Furin mRNA from isolated PBMCs is not useful for facilitating the assessment of disease activity or severity in this patient group.

To our knowledge, this is the first study assessing general protein levels of Furin in plasma and expression of the *FUR* gene in PBMCs in a study population of both patients with pSS and healthy controls. Therefore, relatively little is currently known about the Furin levels in pSS. T-cell-expressed Furin could act as a compensatory mechanism to prevent autoimmune responses and an overly activated immune system orchestrated by IFN-γ. In parallel, an exogenous Furin has been successfully used to harness autoimmunity in experimental animal models (Lin et al., 2012). However, further studies are needed to refine the knowledge of the potential clinical usability of Furin in pSS.

6.3 Furin in RA

The prevalence of chronic inflammatory diseases is rising rapidly, and RA alone is estimated to affect around 1% of all adults (Silman & Pearson, 2002). Because our understanding of the pathological mechanisms that lead to organ damage is still incomplete, currently used diagnostic methods and antirheumatic therapy lack efficacy and specificity. Hence, RA attributes a significant economic burden on our society. There is an increasing need for specific biomarkers for rheumatic diseases, with a particular reference to ones that would allow early detection and identification of patients who benefit from anticytokine treatment. Further, conventional inflammatory markers, such as CRP, do not accurately reflect the disease activity, which complicates the evaluation of the efficacy of a given therapeutic strategy (Orr et al., 2018). As discussed earlier, Furin is an important regulatory factor in both cellular homeostasis and pathogenesis of various diseases. It is shown to regulate the

bioavailability of anti-inflammatory TGF-β1 and appears to have a key role in restraining autoimmune responses and maintaining general immune tolerance (Oksanen et al., 2014; Pesu et al., 2006, Pesu et al., 2008). Previous studies have reported elevated Furin levels in the synovial pannus in RA and salivary gland biopsies in pSS (Lin et al., 2012). In addition, Furin has been found to be upregulated in patients with SLE (Wu et al., 2016). Consistently, our studies of Furin plasma levels and isolated PBMCs indicate that it might have a role in the pathogenesis of autoimmune diseases (I and II). In more detail, upregulated Furin was reported in plasma in patients with suspected infection or underlying rheumatic disease (I) and in isolated PBMCs in pSS (II).

In study III, we investigated Furin levels in patients with active RA and healthy controls to evaluate the potential value of Furin as a biomarker for RA and autoimmunity in general. Because our previous determinations of Furin mRNA from isolated PBMCs failed to present a significant value for clinical use in pSS (II), we wanted to broaden our methods for Furin mRNA determinations in order to achieve a better understanding of their usability in clinical practice. Consequently, here, we isolated mononuclear cells additionally from the synovial fluid samples of patients with active RA to determine local Furin mRNA expression in inflamed joints. To our knowledge, this was the first study to evaluate Furin expression in tissue-infiltrating leukocytes in RA. Our analyses showed that Furin mRNA expression was significantly elevated in active RA, suggesting that Furin is upregulated in two different rheumatic disease types. However, further research is required to evaluate if Furin is upregulated in rheumatic diseases generally.

In the next section of study III, we assessed how Furin levels correlate with the demographic and clinical parameters in RA to evaluate its potential role in diagnosis and treatment planning. Our findings showed that elevated Furin mRNA expression is associated with high-dose prednisolone treatment, increased levels of conventional inflammation markers CRP and ESR, and a greater HAQ disability index. These findings suggest that, contradictory to pSS, a high Furin level is associated with more active and severe RA. This is somewhat surprising, as Furin is known to generally promote peripheral tolerance and restrain proinflammatory cytokine secretion (Cordova et al., 2016; Pesu et al., 2008). Further, previous studies have argued for a protective role of Furin in arthritis (Lin et al., 2012). Consistently, Furin inhibition has been demonstrated to promote an invasive phenotype of synoviocytes in patients with RA Our present observation of increased Furin levels in severe RA may be explained as a compensatory attempt to suppress excessive inflammation. Analogously, previous studies have reported increased levels of other anti-

inflammatory agents as well, including IL-10 and TGF-β, in active RA (McInnes, Buckley, & Isaacs, 2016). However, abundant presence of pro-inflammatory mediators leads to persisting inflammation despite these enforced suppressive functions. Hence, the Furin expression could be high simply because it rises in the immune activation in general.

Finally, we investigated how Furin levels associate with universally expressed plasma cytokines. The analysis showed that patients with elevated Furin levels also tend to have elevated plasma levels of other global cytokines, most significantly proinflammatory IL-2. Because elevated IL-2 levels are a previously reported sign of T cell activation, this finding is consistent with previous studies reporting that Furin is enhanced upon macrophage and T cell activation (Pesu et al., 2008; Turpeinen et al., 2011).

7 CONCLUSIONS AND FUTURE PERSPECTIVES

In the present study, the expression levels of a PCSK enzyme Furin were investigated in different IMDs, using ELISA and Q-RT-PCR methods. In addition, Furin levels were associated with clinical and demographic parameters to assess the potential value of Furin measurements as a biomarker in clinical practice. For the first time, we demonstrated that Furin plasma levels, at least when determined by particular commercial ELISA kits, cannot be used as a diagnostic or prognostic biomarker for first-aid patients with suspected infection in the ER. Moreover, it brings no value for decision-making in treatment planning.

However, analyses from all three presented studies suggest that Furin expression is elevated in autoimmune diseases. We also showed that Furin is upregulated in different rheumatic disease types, more specifically in pSS and RA. Interestingly, our results show contradictory suggestions of the association between Furin and the pathogenesis of these diseases. Investigations of Furin expression in patients with pSS indicated a protective role of Furin in the pathogenesis of pSS, as Furin upregulation was related to signs of somewhat lower disease activity. In contrast, an elevated Furin expression was associated with more severe and treatment-resistant RA. One possible explanation for these rather contradictory results could be that Furin has a disease-specific function. In other words, because Furin cleaves both anti-inflammatory (e.g., TGF-β1) and proinflammatory (e.g., TNF family cytokines and tumor necrosis factor-alpha converting enzymes) factors, its final effects on the immune system can differ greatly on the basis of pathological mechanisms of the diseases (Thomas, 2002). However, further studies and more sensitive Furin measurements with larger study groups are needed to investigate the clinical relevance of presented findings and to refine the potential value of Furin as a biomarker for autoimmune diseases.

Here, we have shown that the used commercial ELISA kits clearly lack sufficient sensitivity and fail to detect low Furin levels in human plasma samples. However, alternative Furin measurement technologies, such as determining Furin activity in fluidic samples using fluorogenic substrates, could yield different results. Importantly, PCSK activity measurements should be optimized to serum or plasma and it is not specific for Furin. In addition, other plasma proteins may interfere with

activity measurements because the current functionality is only based on in vitro experiments (Cork et al., 2012). We have also shown that the use of the Q-RT-PCR method can generate additional information on Furin levels independently of ELISA measurements. We did not face any problems in determining Furin mRNA levels from isolated mononuclear cells, but because of the small sample size, the fundamental functionality should be further tested on a larger patient group.

A greater focus on investigating new methods for measuring Furin expression could reveal new aspects for the clinical usability of Furin. One of the newest detection methods is the proximity extension assay technique with the O-LINK reagent kit, consisting of two highly specific oligonucleotide-labelled antibodies. They form ligations with their target proteins in plasma and can be further quantified using the Q-RT-PCR method (Assarsson et al., 2014). The O-LINK method has recently been reported to be successful in detecting Furin levels in patients with diabetes mellitus, and postural orthostatic tachycardia syndrome (Fernandez et al., 2018; Spahic et al., 2019). Altogether, novel methods for the detection of Furin can provide better ways to understand the fundamental nature of this ubiquitous endoprotease and its value as a biomarker for distinct IMDs.

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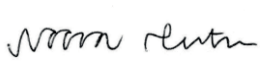

Noora Ranta

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10 ORIGINAL PUBLICATIONS

PUBLICATION I

The Plasma Level of Proprotein Convertase FURIN in Patients with Suspected Infection in the Emergency Room: A Prospective Cohort Study

Ranta Noora, Turpeinen Hannu, Oksanen Anna, Hämäläinen Sanna, Huttunen Reetta, Uusitalo-Seppälä Raija, Rintala Esa, Aittoniemi Janne, Pesu Marko

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The Plasma Level of Proprotein Convertase FURIN in Patients with Suspected Infection in the Emergency Room: A Prospective Cohort Study

N. Ranta*,†, H. Turpeinen*, A. Oksanen*, S. Hämäläinen*, R. Huttunen‡, R. Uusitalo-Seppälä§, E. Rintala¶, J. Aittoniemi** & M. Pesu*,**,††

*Immunoregulation, BioMediTech, University of Tampere, Tampere, Finland; †School of Medicine, University of Tampere, Tampere, Finland; ‡Department of Infectious Diseases, Tampere University Hospital, Tampere, Finland; §Department of Infectious Diseases, Satakunta Central Hospital, Pori, Finland; ¶Department of Hospital Hygiene & Infection Control, Turku University Hospital, Turku, Finland; **Fimlab Laboratories, Tampere, Finland; and ††Department of Dermatology, Tampere University Hospital, Tampere, Finland

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Correspondence to: M. Pesu, MD, PhD, Immunoregulation, BioMediTech, University of Tampere, Biokatu 8, FI-33520 Tampere, Finland. E-mail: marko.pesu@uta.fi

Abstract

There is an increasing need for novel biomarkers that enable better diagnostic and prognostic stratification of patients with suspected infection. A proprotein convertase enzyme FURIN is upregulated upon immune cell activation, and it promotes infectivity by cleaving and activating pathogens. In this study, we determined FURIN levels in plasma using ELISA from 537 patients that were admitted to emergency room with suspected infection. Patients were sorted to high- and low-level FURIN groups with a cut-off level of 370 pg/ml. The study cohort included five diagnostic groups: Group 1, no systemic inflammatory response syndrome (SIRS, $n = 59$ patients); Group 2, bacterial infection without SIRS ($n = 67$); Group 3, SIRS, but no bacterial infection ($n = 308$); Group 4, sepsis without organ failure ($n = 308$); and Group 5, severe sepsis ($n = 49$). Statistically significant associations were not found between the plasma level of FURIN and the prevalence of sepsis ($P = 0.957$), diagnostic group of a patient ($P = 0.737$) or the bacteria in blood culture ($P = 0.499$). Additionally, the concentration of FURIN did not predict the severity or case fatality of the infectious disease. However, statistically significant associations were found between high plasma level of FURIN and diagnosed rheumatic disease ($P < 0.001$) as well as with the prevalence of non-smokers ($P = 0.034$). Thus, albeit the plasma level of FURIN does not predict the severity of infectious disease, it may be of use in the diagnostics of autoimmune diseases.

Introduction

Sepsis is a major cause of mortality. In severe sepsis, mortality rises up to 60–70% [1, 2]. Potentially fast disease progression is the main reason why early diagnosis and quick adequate treatment are extremely important in this condition. Even an hour-long delay in adequate antimicrobial treatment may have an adverse impact on prognosis [3, 4].

Currently used biomarkers for the diagnosis of sepsis are C-reactive protein (CRP), blood leucocytes and procalcitonin (PCT) [5, 6]. None of these biomarkers are suitable for the early stages of sepsis; they can be used for confirmation, but not for exclusion of the diagnosis. For example, CRP levels begin to rise only after 12 h of the early signs of sepsis and it is not a specific marker of bacterial infection [7, 8].

The proprotein convertase subtilisin/kexin (PCSK) enzymes belong to the subtilisin superfamily of serine endoproteases, which convert immature precursor proteins into the biologically functional units by catalysing post-translational site-specific hydrolytic cleavage [9, 10]. FURIN is the first found and therefore the most studied mammalian proprotein convertase. This ubiquitously expressed enzyme is located mainly in the trans-Golgi network (TGN) of the secretory pathway. In addition, FURIN cycles between TGN and the cell surface via the endosomal system, and a proportion of it is secreted to the extracellular space [11, 12]. Because FURIN is widely expressed, its activity regulates the maturation of numerous precursor proteins. Its substrates include various growth factors and their receptors, enzymes, hormones, cytokines, serum proteins, as well as extracellular matrix proteins [10, 11]. In addition to FURIN's crucial function in the maintenance of cellular homoeostasis, it is also involved in the pathogenesis of several diseases. For example, increased FURIN levels are associated with

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aggressive cancers and metastatic activity [11], and it is highly expressed in chronically inflamed tissues in rheumatoid arthritis (RA) and atherosclerosis [13, 14]. FURIN has also a significant role in the activation of viruses and bacterial pro-toxins. These pathogens include bird flu (AH5N1), HIV and Ebola viruses as well as the toxins of *Bacillus anthracis* and *Clostridium botulinum* [12].

Because FURIN is essential for the embryogenesis, our understanding on its cell-type-specific *in vivo* function is incomplete [13]. However, we have previously shown that FURIN is profoundly upregulated in T helper type 1 (Th1) cells via the IL-12/Stat4 pathway and by T cell receptor-mediated signalling [15]. T cell-expressed FURIN regulates the Th cell polarization and the peripheral immune tolerance primarily by controlling the functional maturation of anti-inflammatory cytokine transforming growth factor-beta (TGF-β1) [15–17]. In macrophages, the expression and secretion of FURIN is induced by lipopolysaccharide (LPS) [18, 19], and a FURIN-like enzyme controls the quantity and activation of the human Toll-like receptor 7 (TLR7) [20]. In contrast, relatively little is known about the FURIN levels in human infections, but an elevated serum concentration has been reported in chronically *Salmonella typhi*-infected patients [18].

The fact that FURIN is upregulated upon immune cell activation and its key regulatory role in host defence prompted us to evaluate the usability of plasma FURIN as an early stage infection biomarker. Specifically, we analysed whether the proprotein convertase FURIN levels in plasma could be useful in predicting the severity and case fatality of infection in a large, randomly selected cohort of patients that were admitted the emergency room with suspected infection.

Methods

Study population. The study population was recruited in the Satakunta Central Hospital, Finland within a 14-month study period in 2004 to 2005 [6, 21–23]. Satakunta Central Hospital is a secondary care hospital with 350-beds serving a population of 240 000 inhabitants. There are no other hospitals with an emergency room and an intensive care unit (ICU) in the Satakunta area. All of the involved patients were adults, who had been admitted to the emergency room with a suspected infection status and been drawn a blood culture sample. The study was approved by the Ethical Review Board of the Satakunta Hospital District. Written informed consent was obtained from patients or close relatives. To ensure written informed consent and interview within 24–48 h, only patients admitted between Sunday 7 a.m. and Wednesday 3 p.m. were enrolled [23].

The plasma samples were taken simultaneously with the blood culture samples and collected in two 10-ml EDTA tubes. The EDTA tubes were kept on ice before the centrifugation in 2500 G-force for 10–15 min. The samples were then transported in 1 to 2 ml aliquots to CryoPure® (Sarstedt, Germany) tubes and further stored at −70 °C [23].

The information for the statistical analysis was obtained from an interview held by a researcher or a research nurse within 24–48 h after the admission. Highest body temperature, lowest blood pressure, highest pulse and the respiratory rate were measured daily during the first week of hospitalization. In addition, symptoms and clinical signs, Glasgow coma scale, risk factors for sepsis, underlying disease, diagnosis at admission, prospective organ failure (cardiovascular, haematological, hepatic, renal, respiratory or central nervous system), universal case fatality and the case fatality caused by sepsis were documented in ICU and in the ward. Other information such as the potential reason and the cause of the infection and trauma together with the final diagnose was checked from patient's medical records. Follow-up surveys were conducted by phone 3 months and one year after patient's admission. Before initiating the study, a pre-evaluation of the target population was conducted to ensure the representativeness of the cohort. This assessment covered 1551 consecutive patients from whom blood had been collected in the emergency department for blood culture. The rate of positive blood culture in the pre-evaluation was 8.3%, and case fatality by day 28 after admission was 6.7%. No significant differences in age, gender, rate of positive blood culture or mortality were noted between patients admitted on study days and those admitted on other days, or between the study and the pre-evaluation populations. [23]

In total, blood samples were taken from 609 patients. Fifty-five samples were removed because of the patient's reluctance to participate in the research, and 17 samples were left out from other reasons: 1 missing, 11 unclear results, 3 with SIRS and multi-organ failure but no bacterial infection and two samples ran out. As a result, the final cohort comprised of 537 patients [23].

Measurement of plasma FURIN levels. The concentration of proprotein convertase FURIN was determined from EDTA plasma samples using a commercial Human FURIN enzyme-linked immunosorbent assay (ELISA) kit (Sigma-Aldrich®, USA) according to manufacturer's instructions. The reliable detection limit of the assay was determined to be 370 pg/ml, and the concentrations below that were considered as low/absent. In general, all samples were analysed once, but analysis was replicated with a higher dilution for the samples showing exceptionally high FURIN levels. The patients were stratified into low and high plasma FURIN groups with a cut-off at 370 pg/ml.

Statistical analysis. The detected FURIN concentrations were transferred to the SPSS Statistics for Windows software (IBM, version 22) for statistical analyses. The study population was divided into five diagnosis groups

based on the American Collage of Chest Physicians/Society of Critical Care Medicine Consensus Conference definitions of the severity of the infection stage [24].

Nonparametric independent-samples *t*-test for independent samples or analysis of variance was used with normally distributed variables, and Mann–Whitney *U*-test or Kruskal–Wallis test for the rest. Two binomial variables were analysed with chi-square or Fisher's exact test. Linear regression and logistic regression were used to examine the effect of potential confounders. The Kaplan–Meier and the log-rank test were used to investigate the connection between the FURIN levels and the case fatality.

The common significance levels were used to determine the importance of the outcomes: $P < 0.05$ illustrated a statistically significant result, and $P < 0.001$ was considered as a statistically highly significant discovery.

Results

The demographic data of the study population ($n = 537$), underlying diseases as well as the infection-associated clinical parameters during the first 28 days, are presented in Table 1. The plasma FURIN levels were measured from all samples as described in methods. The assay standard curve was prepared using serial dilutions of recombinant FURIN protein, and a reliable linearity was detected only with FURIN levels above 370 pg/ml, which was thus determined as cut-off for high FURIN levels in plasma. The cohort was stratified into five study groups on the basis of ACCP/SCCM Consensus Conference definitions (Table 2) [24] and further divided into low and high FURIN samples. In the five different groups, high FURIN levels were detected in 14.9% - 22.0% of samples, with lowest percentage of high FURIN samples in bacterial infection, no SIRS group (Group 2) and highest in no SIRS, no bacterial infection group (Group 1). However, differences in the prevalence of high FURIN levels between different groups were not significant ($P = 0.737$).

We next assessed whether high FURIN level in plasma is associated with patients' demographic characteristics or infection-associated clinical parameters (Table 3). Although elevated FURIN levels have been found in malignancies and atherosclerosis [11, 13, 25], there were no statistically significant associations with high FURIN plasma levels and the prevalence of solid cancer ($P = 0.606$) or cardiovascular diseases ($P = 0.204$). However, in keeping with the elevated FURIN expression in inflamed tissues in RA [14], FURIN plasma concentrations over 370 pg/ml were associated highly significantly with a diagnosed rheumatic disease. Specifically, only 7.0% of the patients in the group with lower levels of FURIN were suffering from rheumatic diseases in contrast to 18.5% in the high FURIN level group ($P < 0.001$). The plasma level of FURIN was over 370 pg/ml in 40% of arthritis patients compared to only 18% in the cohort without rheumatic disease (data not shown). Interestingly, albeit tobacco smoking places a burden on the immune system, and could thus be envisioned to increase FURIN expression through the immune cell activation, we also found a significant association between current smokers and low FURIN plasma levels ($P = 0.034$).

In contrast, during the first 28 days, FURIN plasma levels did not significantly associate with any of the investigated clinical parameters including multi-organ failure ($P = 0.421$) or the occurrence of sepsis ($P = 0.957$); also there was no statistically significant

Table 1 Demographic and clinical characteristics of the study population ($N = 537$).

	N	Percentage
Characteristic		
Age, median (range)	64.2 (18–100)	
Gender (male)	310	57.7
Obesity (BMI[a] ≥30 kg/m^2), median (range)	27.0 (14.7–67.6)	
Alcohol abuse[b]	25	4.7
Smoking (current smoker)	126	23.5
Diabetes (types 1 and 2)	81	15.1
Solid cancer	78	14.5
Malignancy (solid or haematological)	95	17.7
Rheumatic disease	50	9.3
Chronic renal insufficiency[c]	18	3.4
Cardiovascular disease[d]	289	53.8
Continuous cortisone treatment[e]	59	11.0
Clinical parameters (day 0–28)		
Case fatality (d 28)	33	6.1
Case fatality (d 90)	58	10.8
Case fatality (1 year)	112	20.9
ICU[f] stay needed	42	7.8
Hypotension[g]	28	5.2
DIC[h]	8	1.5
Decreased GCS[i]	60	11.2
Mechanical ventilation	14	2.6
C-PAP/bi-PAP[j]	22	4.1
Sepsis + organ dysfunction	49	9.1
MOF[k]	10	1.9

Demographics are reported as a number and percentage except age and obesity which are reported as median and range.
[a]Body mass index, data available on 390 patients.
[b]Alcoholism was diagnosed or patient had previously been treated for alcohol-induced disease.
[c]Plasma creatinine concentration continually more than 170 μmol l^{-1} (five patients underwent chronic dialysis treatment).
[d]Continuous medication for cardiovascular disease (including hypertension and arteriosclerosis).
[e]Continuous systemic cortisone treatment (daily dose >10 mg oral prednisolone).
[f]Intensive care unit.
[g]Systolic blood pressure < 90 mmHg or a reduction of 40 mmHg from baseline. No response to 500 ml intravenous fluid replacement.
[h]Disseminated intravascular coagulation.
[i]Glasgow coma scale <15.
[j]Continuous positive airway pressure/bilevel positive airway pressure.
[k]Multi-organ failure.

Table 2 The distribution of emergency room patients stratified by diagnosis group into low or high FURIN levels.

		FURIN < 370 pg/ml (N = 429)		FURIN ≥ 370 pg/ml (N = 108)	
Diagnosis group	Criteria	n	Percentage	n	Percentage
1. No SIRS, no bacterial infection (N = 59)	Patients with no SIRS[a] (less than two SIRS criteria ±24 h) or documented[b] or probable[c] bacterial infection	46	78.0	13	22.0
2. Bacterial infection, no SIRS (N = 67)	Patients with documental or probable bacterial infection, but no SIRS (less than two SIRS criteria ±24 h)	57	85.1	10	14.9
3. SIRS, no bacterial infection (N = 54)	Patients with SIRS (at least two SIRS criteria ±24 h), but no documental or probable bacterial infection	45	83.3	9	16.7
4. Sepsis (N = 308)	Patients with sepsis (SIRS and documented or probable bacterial infection but no dysfunction due to sepsis)	242	78.6	66	21.4
5. Severe sepsis (N = 49)	Patients with severe sepsis (sepsis with signs of organ failure, that is distributed perfusion, metabolic acidosis, oliguria or neurological disorders)	39	79.6	10	20.4

Differences between five groups were studied using the chi-square test (P = 0.737).

[a]Systemic inflammatory response syndrome (SIRS): at least two of the following conditions. 1. Temperature >38 °C or <36 °C; 2. Heart rate >90 beats per min; 3. Respiratory rate >20 breaths per min or partial pressure of carbon dioxide in arterial blood [$PaCO_2$] <32 mmHg (4.3 kPA). 4. White blood cell count >12 × 109 l^{-1} or >10% immature (band) forms.

[b]Documented bacterial infection: microbiologically confirmed bacterial infection (either pathogenic bacterial growth in blood culture or in normally sterile tissue or the same usually less pathogenic bacterium, for example *Staphylococcus epidermidis*, in two different samples).

[c]Probable bacterial infection: a clinician suspected bacterial infection and either infection focus was confirmed or antimicrobial treatment was started and the response to treatment supported bacterial infection.

connection to a 28-day case fatality when examining patients with sepsis as a direct cause of dead or a contributing factor on patient's decease (P = 0.463). In conclusion, the current data imply that assessing FURIN plasma levels does not have value as a diagnostic marker for patients with a suspected infection.

To investigate whether FURIN plasma levels could associate with the Gram staining results of the blood culture samples, we divided the study population into four groups with the alternatives of no growth, Gram-positive, Gram-negative and mixed infection (Table 4); no significant differences in FURIN levels were observed (P = 0.351). To explore the more accurate distribution of various bacterial species into the two FURIN plasma levels, the blood culture results were further divided into the Gram-positive and Gram-negative bacterial species (Table 5). Gram-negative *Escherichia coli* (n = 11) and Gram-positive *Streptococcus pneumoniae* (n = 10) were noticeably the most common causes of a systemic bacterial infection among our study cohort. However, the findings again indicated that FURIN plasma levels measured simultaneously with the blood culture sampling are not informative considering the bacterial strain causing the infection.

To estimate the potential use of FURIN as a marker for a patient's prognosis in predicting the severity and the case fatality, we constructed two survival curves. First, we examined the overall case fatality during the 1-year study period (Fig. 1). The plasma level of FURIN failed to prognosticate the case fatality of an infection in a 1-year lasting follow-up period (P = 0.742). Finally, we wanted to clarify the possible prognostic value of FURIN concentrations especially among the patients who had been diagnosed with sepsis at the emergency room. According to the fact that basically all of the sepsis-related deaths occur in the first four weeks since the symptoms had started, we limited the follow-up time into 28 days. The cases where sepsis was not a direct cause of dead or did not contribute to patient's decease were censored (n = 13) from the analysis (Fig. 2). The statistical significance was not found between the survival probability of septic patients and FURIN concentration level (P = 0.898).

Discussion

We examined the usability of the ubiquitously expressed proprotein convertase FURIN as an infection biomarker by determining its plasma concentrations in the study cohort of 537 patients admitted to the emergency room with a suspected infection. The present results revealed that FURIN cannot be used as a diagnostic biomarker at the emergency room in the early phase of an infection, even though it has earlier been associated with various inflammatory conditions [12, 13, 15, 17–20]. There were also no statistically significant associations between the FURIN plasma levels and the Gram staining or the bacterial species of the blood cultures. The concentration of FURIN neither associated with the case fatality or the severity of the infection in our study cohort. In contrast, we found that patients with a rheumatic disease and patients that were not currently smokers had significantly more frequently high plasma concentrations of FURIN.

Table 3 The distribution of patients' demographic characteristics and clinical parameters into the two FURIN levels (N = 537).

	FURIN < 370 pg/ml (N = 429)		FURIN ≥ 370 pg/ml (N = 108)		
	N	Percentage	*N*	Percentage	*P*-value
Characteristic					
Age, median (range)	63.82 (18–100)		66.38 (20–94)		0.618
Gender (male)	246	57.35	64	59.3	0.719
Obesity (BMI[a] ≥ 30 kg/m^2), median (range)	27.20 (14.7–62.5)		26.67 (15.1–67.6)		0.320
Alcohol abuse[b]	22	5.1	3	2.8	0.300
Smoking (current smoker)	109	25.4	17	15.7	**0.034**
Diabetes (types 1 and 2)	68	15.9	13	12.0	0.322
Solid cancer	64	14.9	14	13.0	0.606
Malignancy (solid or haematological)	79	18.4	16	14.8	0.381
Rheumatic disease	30	7.0	20	18.5	**<0.001**
Chronic renal insufficiency[c]	16	3.7	2	1.9	0.333
Cardiovascular disease[d]	225	52.4	64	59.3	0.204
Continuous cortisone treatment[e]	42	9.8	17	15.7	0.077
Clinical parameters (day 0–28)					
Case fatality (day 28)	28	6.5	5	4.6	0.463
Case fatality (day 90)	49	11.4	9	8.3	0.355
Case fatality (1 year)	90	21.0	22	20.4	0.889
ICU[f] stay needed	30	7.0	12	11.1	0.154
Hypotension[g]	23	5.4	5	4.6	0.760
DIC[h]	7	1.6	1	0.9	0.588
Decreased GCS[i]	50	12.2	10	9.6	0.460
Mechanical ventilation	11	2.6	3	2.8	0.901
C-PAP/bi-PAP[j]	16	3.7	6	5.6	0.392
Sepsis + organ dysfunction	39	9.1	10	9.3	0.957
MOF[k]	9	2.1	1	0.9	0.421

Demographics are reported as a number and percentage in the particular concentration of FURIN

Except age and obesity which are reported as median and range.

Statistically significant p values are bolded.

[a]Body mass index, data available on 390 patients.

[b]Alcoholism was diagnosed or patient had previously been treated for alcohol-induced disease.

[c]Plasma creatinine concentration continually more than 170 μmol l^{-1} (five patients underwent chronic dialysis treatment).

[d]Continuous medication for cardiovascular disease (including hypertension and arteriosclerosis).

[e]Continuous systemic cortisone treatment (daily dose > 10 mg oral prednisolone).

[f]Intensive care unit.

[g]Systolic blood pressure < 90 mmHg or a reduction of 40 mmHg from baseline. No response to 500 ml intravenous fluid replacement.

[h]Disseminated intravascular coagulation.

[i]Glasgow coma scale < 15.

[j]Continuous positive airway pressure/bilevel positive airway pressure.

[k]Multi-organ failure.

Table 4 The distribution of the Gram staining of bacteria in blood culture findings into the FURIN levels.

	FURIN < 370 pg/ml (N = 429)		FURIN ≥ 370 pg/ml (N = 108)	
Blood culture finding	*N*	Percentage	*N*	Percentage
No growth (N = 485)	389	80.2	96	19.8
Gram-positive (N = 28)	20	71.4	8	28.6
Gram-negative (N = 16)	12	75.0	4	25.0
Mixed (N = 7)	7	100.0	0	0.0

Differences between five groups were studied using the chi-square test (P = 0.351).

Table 5 The distribution of different bacterial species measured from blood cultures into two FURIN levels.

	FURIN < 370 pg/ml *N*	FURIN ≥ 370 pg/ml *N*	Total
Gram-positive bacteria			
Stafylococcus aureus	3	1	4
Other Stafylococcus	2	2	4
Streptococcus pneumoniae	8	2	10
Other Streptococcus	4	1	5
Listeria monocytogenes	0	1	1
Anaerobic Gram-positive bacteria/bacillus	2	1	3
Gram-negative bacteria			
Escherichia coli	9	2	11
Pseudomonas	1	0	1
Other Enterobacterium[a]	3	0	3
Neisseria meningitidis	0	1	1
Klebsiella	4	1	5
Anaerobic Gram-negative bacteria/bacillus	1	0	1

Differences between values in the table were studied using the chi-square test (P = 0.499).

[a]*Salmonella, Morganella, Proteus, Pantotea.*

The strong association of the elevated plasma FURIN in patients with a rheumatic disease is consistent with the earlier findings showing upregulated FURIN in the inflamed joints of patients with RA [14]. The role of FURIN in autoimmune diseases is not fully understood, but its expression in T cells is critical for the functional maturation of pro-TGF-β1 and adequate function of CD4+ Foxp3+ T regulatory cell-dependent immune tolerance [17]. Moreover, administration of the recombinant FURIN alleviates the inflammation in an experimental collagen-induced arthritis (CIA) mouse model [14]. Theoretically, elevated FURIN levels in plasma could thus reflect an attempt to harness overly active host defence by the activation of anti-inflammatory responses. However, as T cell-expressed FURIN also inhibits Th2 responses and

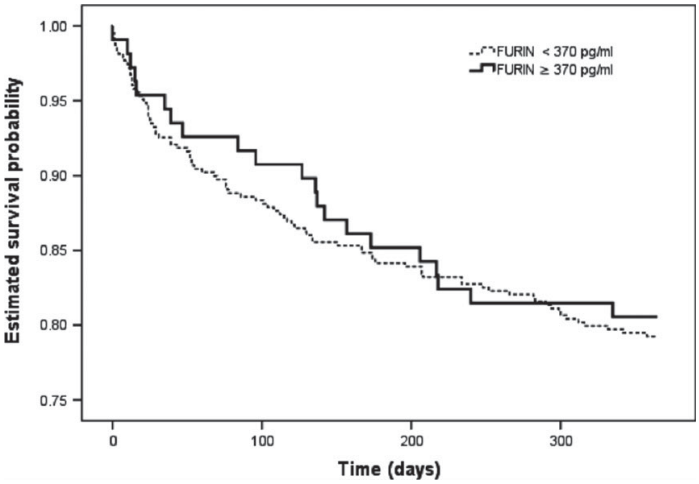

Figure 1 The survival curve measuring the correlation between low (N = 429) and high (N = 108) FURIN levels and the case fatality among the entire study population explored during the 1-year follow-up (P = 0.742).

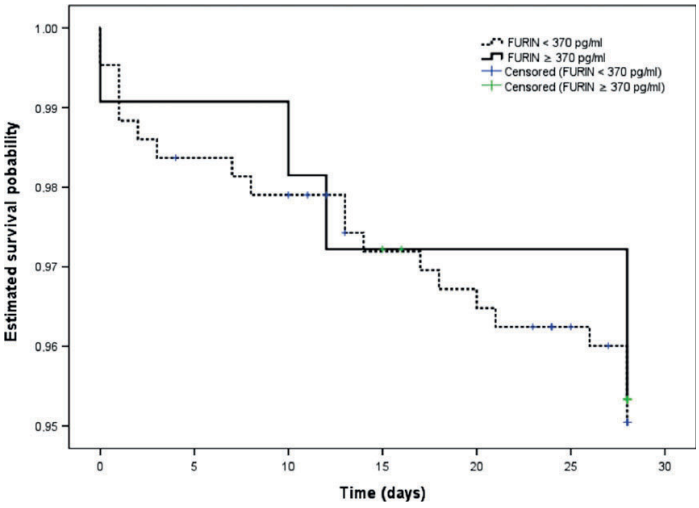

Figure 2 The survival curve measuring correlation between low (N = 429) and high (N = 108) FURIN levels and the case fatality among the patients with the septic infection explored during the 28-day follow-up (P = 0.898). Deaths for which the sepsis found in the emergency room did not influence or was not the direct cause of the expiration are censored from the curve (N = 13).

promotes Th1-type inflammation, further studies are clearly required to fully understand the immunoregulatory role of FURIN in plasma [16]. In any case, our current finding calls for a further assessment of the value of plasma FURIN levels as a biomarker in autoimmune diseases.

A statistically significant association was also found between the FURIN concentrations below 370 pg/ml and the habit of smoking. This is somewhat counterintuitive as smoking is generally regarded to evoke a chronic inflammatory state in the body involving the expansion in the quantity of proinflammatory cytokines [26]. One possible explanation could be that tobacco-related substances in plasma interfere with the FURIN measurement and thus falsify the data. Therefore, analysing FURIN mRNA or protein expression directly in the lungs could give more reliable data on the effect of smoking on FURIN levels.

To our knowledge, this was the first study assessing the usefulness of proprotein convertase FURIN as a diagnostic biomarker for the infections. The strengths of our study were a randomly selected and comprehensive study cohort, a high quality of information collection and the follow-up together with the broad data on patients' demographic and clinical characteristics. The wide data including the information starting from the underlying diseases and medications as well as received treatment during the hospital stay and rehabilitation occurred within 1-year study time, allowed us to undergo multifarious statistical

analysis. The information bias and random error were minimized by taking the plasma samples for the study simultaneously with the samples taken for the diagnostic tests and defining the exact timing for the interviews. This reduced the potential occurrence of both the selection and the information bias making the presented results largely generalizable to the infectious patients in the Finnish population.

The sensitivity of the FURIN ELISA kit did not allow measurement of low FURIN concentrations, but forced us to stratify the samples into low and high FURIN groups. The transformation of the variable from scale to ordinal caused a loss of information and moreover led to the reduction of a statistical power of our data. In addition to us, also other research teams have reported the sensitivity of FURIN antibody assays as problematic [27]. A more sensitive method for the determination of proprotein convertases in plasma could be a direct measurement of the FURIN's activity using a fluorogenic substrate [28]. The downside is that the concerned technique does not distinguish PCSK enzymes from each other.

To determine the FURIN plasma levels in a healthy population, we performed a small-scale pilot study with six healthy volunteers (data not shown). All these samples showed FURIN levels below the cut-off used in this study, but a larger cohort and a more sensitive measurement technique are clearly needed to obtain reliable information on the range of normal plasma FURIN. This may also result in reinterpretation of the current data; if the starting levels of FURIN in healthy individuals are highly variable, the potential rise during the infection would have remained unnoticed even if it occurred consistently with every patient. Also, due to lack of sensitivity of the used ELISA kit, it cannot be excluded that infection-associated changes below 370 pg/ml may have a diagnostic value.

In conclusion, we have shown that plasma FURIN levels assessed with the current method cannot be used as a diagnostic biomarker in patients with suspected infection and fail to predict case fatality or severity. However, even a low sensitivity ELISA measurement implies that plasma FURIN levels may associate with autoimmunity.

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PUBLICATION II

Proprotein convertase enzyme FURIN is upregulated in primary Sjögren's syndrome

Ranta Noora*, Valli Atte*, Grönholm Anna, Silvennoinen Olli, Isomäki Pia, Pesu Marko*, Pertovaara Marja*

*) Equal contribution

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Proprotein convertase enzyme FURIN is upregulated in primary Sjögren's syndrome

N. Ranta[1,2], A. Valli[1], A. Grönholm[1,2], O. Silvennoinen[1,3], P. Isomäki[4,5], M. Pesu[2,5,6], M. Pertovaara[4,5]

[1]Faculty of Medicine and Life Sciences, University of Tampere; [2]BioMediTech, Tampere; [3]Fimlab Laboratories, Tampere; [4]Department of Internal Medicine, Centre for Rheumatic Diseases, Tampere University Hospital; [5]Faculty of Medicine and Life Sciences, University of Tampere; [6]Department of Dermatology, Tampere University Hospital, Tampere, Finland.

Noora Ranta, Medical student*
Atte Valli, Medical student*
Anna Grönholm, PhD
Olli Silvennoinen, MD, PhD
Pia Isomäki, MD, PhD
Marko Pesu#, MD, PhD
Marja Pertovaara#, MD, PhD

**N. Ranta and A. Valli contributed equally to this work.*
#M. Pesu and M. Pertovaara contributed equally to this work.

Please address correspondence to:
Dr Marja Pertovaara,
Department of Internal Medicine,
Centre for Rheumatic Diseases,
Tampere University Hospital,
P.O. Box 2000,
FI-33521 Tampere, Finland.
E-mail: marja.pertovaara@fimnet.fi

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ABSTRACT

Objective. *The proprotein convertase enzyme FURIN is a critical regulator of the anti-inflammatory TGFβ-1 cytokine and peripheral immune tolerance. In T cells, FURIN is co-regulated with IFN-γ and thus highly expressed in T helper 1 type cells. Previous studies have demonstrated that FURIN is upregulated in inflammatory conditions, including atherosclerosis, rheumatoid arthritis and systemic lupus erythematosus. Here, we evaluated the levels of FURIN in the plasma and peripheral blood mononuclear cells (PBMCs) of patients with primary Sjögren's syndrome (pSS) and in healthy controls.*

Methods. *FURIN plasma levels were determined by ELISA, and the mRNA expression in PBMCs was quantitated using qPCR. FURIN levels in the plasma were correlated with the clinical and demographic characteristics of the patients.*

Results. *FURIN was found to be significantly upregulated at both the protein and mRNA level in pSS patients compared to healthy controls. In pSS patients, high FURIN protein levels were significantly associated with elevated IFN-γ levels in the plasma as well as a longer duration of sicca symptoms in the eyes. pSS patients with high FURIN levels in their plasma showed a trend towards lower levels of serum beta-2 microglobulin, ESR and a lower systemic disease activity index ESSDAI.*

Conclusion. *The proprotein convertase FURIN is significantly upregulated in pSS. Elevated FURIN levels associate with high levels of the Th1 type cytokine IFN-γ and long duration of dry eye symptoms. Patients with high FURIN levels show signs of lower disease activity suggesting that FURIN might have a protective role in pSS.*

Introduction

Primary Sjögren's syndrome (pSS) is a chronic systemic autoimmune disease characterised by lymphocytic infiltration in the salivary and lacrimal glands and over-activation of B cells leading subsequently to hypergammaglobulinaemia and development of autoantibodies (1, 2). According to current knowledge, the main pathophysiological mechanisms in pSS are mediated through the interferon (IFN) I and IFN II pathways (1, 2). The type II cytokine IFN-γ has been found to be responsible for the gland dysfunctions in Ro/SSA immunised mice. *Vice versa*, a reduction in the level of IFN-γ or IFN-γR seems to inhibit the development of pSS (2).

The proprotein convertase subtilisin/kexin enzymes (PCSKs) activate various immature proteins by catalysing their post-translational site-specific hydrolytic cleavage (3). The ubiquitously expressed PCSK enzyme FURIN catalyses the proteolysis of a large number of substrates with immunoregulatory functions including cytokines, integrins and viral envelope proteins (3-5). FURIN is upregulated in Th1 cells via the IL-12/Stat4 pathway (6). The T-cell-expressed FURIN regulates Th cell polarisation and peripheral immune tolerance by controlling the functional maturation of transforming growth factor beta (TGFβ-1) (3, 6, 7). FURIN is also upregulated in chronic autoimmune inflammation as is seen in patients with rheumatoid arthritis (RA) (8) and systemic lupus erythematous (SLE) (9).

The fact that FURIN is an important regulator of peripheral immune tolerance and highly expressed in Th1-type lymphocytes prompted us to determine the levels of the FURIN protein and mRNA in patients with pSS compared to healthy controls.

Methods

Study population

Peripheral blood (PB) samples were obtained from 16 (14 female and 2 male) patients with pSS that were recruited from the Centre for Rheumatic Diseases at the Tampere University Hospital, Finland. The inclusion criteria were fulfilment of at least four of the revised American-European consensus group criteria for pSS (10), together with a confirmation of an active disease verified with either an ESSDAI >11 (11) or with laboratory tests: erythrocyte sedimentation rate (ESR) >20 mm/h serum immunoglobulin G (IgG) >15 mg/L, serum beta-2 microglobulin >2.2 mg/L or serum complement C4 <0.10 mg/L. The median age of the patients was 53 years (range 32–80 years) and disease duration 11 years (range 0–27 years). Three of the pSS patients used medication during sampling: one had a low-dose of prednisolone, one received prednisolone and hydroxychloroquine and the third was treated with prednisolone and azathioprine. The clinical characteristics of the pSS patients have been described in detail previously (12).

In addition, 14 control plasma samples for FURIN ELISA measurements and 10 PBMC samples for qPCR analyses were collected from anonymous healthy blood donors (Finnish Red Cross Blood Transfusion Service, Tampere, Finland). Median age of the 14 anonymous healthy blood donors (6 female, 8 male) for FURIN ELISA measurements was 50 years (range 21–63 years).

Measurement of plasma FURIN levels

The plasma concentration of FURIN was determined using a commercial Human FURIN Enzyme-Linked Immunosorbent Assay (ELISA) Kit (Sigma-Aldrich®, St. Louis, MO, USA) according to the manufacturer's instructions. The detection limit of the ELISA assay was 123 pg/ml, and concentrations below this were considered to represent absence of the protein. Duplicate samples were analysed to ensure reliable results. The patients were further stratified into low and high plasma FURIN groups by a median plasma FURIN concentration of 2690 pg/ml.

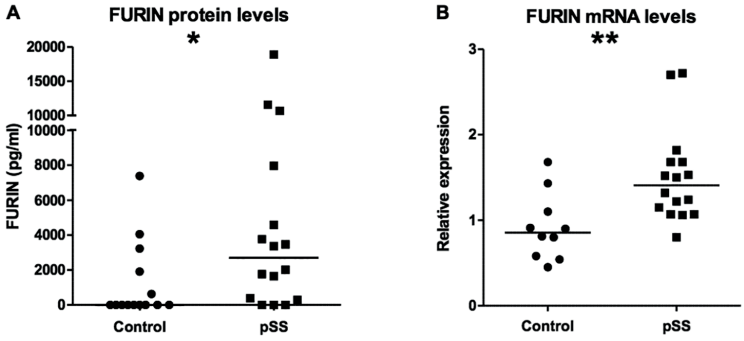

Fig. 1. FURIN levels in patients with primary Sjögren's syndrome (pSS) and in healthy controls. The horizontal lines represent the median values within the groups.
A. Plasma FURIN levels in patients with pSS compared to healthy controls. *p=0.0181.
B. FURIN gene expression in PBMCs from patients with pSS compared to healthy controls. **p=0.0061.

RNA isolation and qRT-PCR analysis

Total RNAs were isolated from PB mononuclear cells (PBMCs). The expression of FURIN (*FURIN*) and the TATA-binding protein (*TBP*, housekeeping) genes were quantified by real-time PCR using the CFX instrument (Bio-Rad, Hercules, CA, USA). Primers: 5'-GGCAAAGCGACGGACTAAAC-3' and 5'-CGTCCAGAATGGAGACCACA-3' for *FURIN* and 5´-GAATATAATCCCAAGCGGTTTG-3´ and 5´-ACTTCACATCACAGCTCCCC-3' for *TBP* (12). The relative FURIN expression was calculated by dividing mean expression values of FURIN measured from triplicated samples by mean expression values obtained for the TBP house-keeping gene.

Statistical analysis

Statistical analyses were performed with SPSS Statistics (IBM, v. 20). The Mann-Whitney U-test was used for comparisons of continuous variables. Correlations were calculated with Spearman's correlation coefficient. Findings were considered statistically significant at $p<0.05$.

Ethical considerations

The study was approved by the Ethical Committee of Tampere University Hospital and conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants.

Results

First, to evaluate whether FURIN is elevated in pSS we measured protein levels in plasma using a commercial ELISA assay. Plasma FURIN levels were significantly upregulated in patients with pSS compared to healthy controls (median 2690 pg/ml vs. 0 pg/ml, p=0.0181, Fig. 1A). Consistent with the plasma data, also FURIN gene expression in PBMCs was significantly higher among patients with pSS compared to the controls (p=0.0061, Fig. 1B).

Next, we examined if the upregulated plasma FURIN associates with clinical or immunological features of patients with pSS. To this end, the pSS patients were divided into high and low-level FURIN groups with a cut-off at the median FURIN concentration 2690 pg/ml (Table I). Patients with high FURIN concentrations showed significantly elevated levels of IFN-γ compared to those with low FURIN concentrations (median 100 pg/ml *vs*. 27.2 pg/ml, p=0.036). Moreover, they had a longer duration of sicca symptoms of the eyes (median 13 years *vs*. 9 years, p=0.035). There was also a statistically significant correlation between plasma FURIN-levels and IFN-γ -levels (Spearmann correlation, r=0.631, p=0.009) and with the duration of sicca symptoms of the eyes (r=0.520, p=0.038). In addition, there was a trend towards lower levels of serum beta-2 microglobulin in pSS patients with high FURIN levels compared to those with low levels

Table I. Clinical and immunological findings in 16 patients with pSS grouped by median plasma FURIN concentrations.

Variable	Plasma FURIN concentration <2690 pg/ml n=8	Plasma FURIN concentration ≥2690 pg/ml n=8	*p*-value
Age, years	52 (40-64)	57 (43-75)	0.563
Duration of sicca symptoms of the eyes, years	7 (3-15)	19 (14-35)	0.035
Duration of sicca symptoms of the mouth, years	10 (6-22)	15 (10-21)	0.461
Disease duration, years	9 (5-17)	13 (6-19)	0.599
ESSPRI, cm	4.37 (3.17-6.33)	4.28 (2.15-5.76)	0.674
ESSDAI	6.50 (4.25-9.75)	3.00 (2.25-7.50)	0.205
Pain-VAS, cm	4.0 (0-66.0)	5.5 (0-16.0)	0.675
PGH-VAS, cm	15.0 (1.0-71.0)	14.5 (2.25-25.0)	0.523
HAQ	0.00 (0-0.38)	0.13 (0-1.10)	0.433
Haemoglobin, g/L	130 (121-136)	137 (126-142)	0.247
ESR, mm/h	29 (11-37)	11 (8-19)	0.138
C-reactive protein, g/L	0.85 (0-2.55)	0.00 (0.00-0.75)	0.277
Serum IgG, g/L	17.8 (16.5-22.3)	16.9 (14.3-20.8)	0.462
Serum IgA, g/L	2.63 (1.78-3.15)	2.12 (0.25-3.15)	0.400
Serum IgM, g/L	1.50 (0.92-2.97)	1.14 (0.61-2.21)	0.600
Serum β2m, mg/L	3.05 (2.80-3.43)	2.30 (2.08-3.08)	0.050
Serum C3, g/L	0.98 (0.84-1.21)	0.98 (0.83-1.15)	1.000
Serum C4, g/L	0.16 (0.11-0.17)	0.14 (0.12-0.18)	0.792
Anti-SSA antibody titre	240 (240-240), n=7	240 (240-240), n=6	1.00
Anti-SSB antibody titre	320 (72-320), n=7	183 (0-320), n=6	0.174
Plasma IL-1 beta, pg/mL	13.9 (1.93-33.6)	18.8 (6.14-24.6)	0.674
Plasma IL-2, pg/mL	21.5 (9.23-112)	67.0 (21.1-108)	0.345
Plasma IL-4, pg/mL	53.1 (42.6-96.7), n=7	146.3 (37.8-347), n=7	0.406
Plasma IL-6, pg/mL	8.04 (4.06-21.4), n=7	13.7 (7.65-31.0)	0.487
Plasma IL-7, pg/mL	15.2 (5.46-21.7)	22.5 (17.4-45.3)	0.115
Plasma IL-10, pg/mL	74.1 (33.7-258)	110.5 (59.0-360)	0.418
Plasma IFN-γ, pg/mL	27.2 (18.1-89.5)	100.0 (78.0-187)	0.036
Plasma TNF-α, pg/mL	7.66 (4.92-16.9)	15.2 (11.3-18.8)	0.141

Statistical analysis: Mann Whitney test. The values are expressed as medians (interquartile range). ESSPRI: EULAR Sjögren's syndrome patient-reported index; ESSDAI: EULAR Sjögren's syndrome disease activity index; VAS: visual analogue scale; PGH: patient's global health assessment; HAQ: Health Assessment Questionnaire; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; β2m: beta-2 microglobulin; IL: interleukin; IFN: interferon; TNF: tumour necrosis factor.

(median 2.30 mg/ml *vs*. 3.05 mg/ml, p=0.050). ESR (median 11 mm/h *vs*. 29 mm/h, p=0.138) and the systemic disease activity index ESSDAI (median 3.00 *vs*. 6.50, p=0.205) were also lower in pSS patients with high FURIN levels (Table I).

Discussion

Previous studies have shown a connection between FURIN and various conditions involving chronic inflammation such as atherosclerosis (5), rheumatoid arthritis (8) and SLE (9). In keeping with these, our recent study with 537 patients with a suspected infection indicated a strong association between high plasma FURIN levels and a history of rheumatic disease (13), yet there was no connection between plasma FURIN levels and demographic features such as age and sex (13). In the present study, we show that both FURIN plasma levels as well as FURIN gene expression are significantly higher in patients with pSS compared to healthy controls, implying that FURIN might have a role in the pathology of pSS.

A significant association was found between high plasma levels of FURIN and elevated plasma levels of the pro-inflammatory cytokine IFN-γ. IFN-γ is generally known to mediate Th1 cell functions, macrophage activation and immunoglobulin class switching, and it has previously been widely associated with systemic autoimmunity. What is noteworthy, IFN-γ and FURIN mRNAs are simultaneously expressed by Th1 cells and they are regulated via the IL-12/Stat4 pathway (6). However, here we demonstrate for the first time that IFN-γ and FURIN also correlate at the protein level in PB.

An association was also found between high FURIN plasma levels and the duration of sicca symptoms of the eyes. In addition, there was a non-significant trend showing that pSS patients with high plasma FURIN levels had milder clinical findings, *i.e*. lower serum beta-2 microglobulin levels, ESR and ESSDAI. As has been previously shown, FURIN is essential for maintaining peripheral tolerance by regulating the maturation of TGFβ-1 (3, 6, 7). A FURIN deficiency leads to the development of less protective Treg cells and a production of overly activated effector T cells with no susceptibility to suppressive actions of wild-type Treg cells (3). A potentially protective role of systemic FURIN has previously been reported in patients with rheumatoid arthritis (8), where it seemed to reverse the Th1/Th2 balance in the joints and enhance the proportion of T regulatory (Treg) cells in the spleen. The protective, symptom-reducing effect of FURIN is additionally supported by the fact that active FURIN has been shown to restrain the production of proinflammatory cytokines in macrophages (14). However, the immunoregulatory role of FURIN is multifaceted. While it seems evident that FURIN exerts an important anti-inflammatory function, it can also promote pro-inflammatory cytokines including the central pSS cytokine BAFF (5).

Elevated levels of FURIN have previously been reported in salivary glands of pSS patients (15). To our knowledge, this is the first study quantitating the protein levels of FURIN in plasma and the expression of FURIN mRNA in PBMCs in patients with pSS. In conclusion, we demonstrate that both plasma FURIN levels as well as FURIN gene expression in PBMCs are higher in patients with pSS compared to healthy controls. In addition, a trend towards somewhat milder clinical findings in patients with high plasma levels of FURIN was observed. These findings are consistent with previous studies on other inflammatory diseases and might reflect a potentially protective role of FURIN in patients with pSS by preventing the autoimmune responses and overly activated immune system

orchestrated by IFN-γ. In experimental models, exogenous FURIN has been successfully used to harness autoimmunity (8). Taking into account the clinical diversity of pSS, it would be important to analyse FURIN levels in subgroups of pSS patients with various extraglandular symptoms and in patients classified by disease duration. Therefore, further studies with more pSS patients are needed to confirm the current findings and to refine our knowledge of the possible clinical feasibility of targeting FURIN in pSS.

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PUBLICATION III

Increased expression of the proprotein convertase enzyme FURIN in rheumatoid arthritis

Valli Atte*, Ranta Noora*, Grönholm Anna, Silvennoinen Olli, Pesu Marko*, Isomäki Pia*

*) Equal contribution

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Increased expression of the proprotein convertase enzyme FURIN in rheumatoid arthritis

A Valli[1*], N Ranta[1,2*], A Grönholm[1,2], O Silvennoinen[1,3], M Pesu[1,2,4**], P Isomäki[1,5**]

[1]Faculty of Medicine and Life Sciences, University of Tampere, Tampere, Finland
[2]BioMediTech, Tampere, Finland
[3]Fimlab Laboratories, Tampere, Finland
[4]Department of Dermatology, Tampere University Hospital, Tampere, Finland
[5]Department of Internal Medicine, Centre for Rheumatic Diseases, Tampere University Hospital, Tampere, Finland

Objective: FURIN is a proprotein convertase enzyme that inhibits the proinflammatory function of T cells and myeloid cells. Elevated FURIN expression levels have been reported in immune-mediated diseases, such as primary Sjögren's syndrome. Here, we investigated the levels of FURIN in peripheral blood (PB) and synovial fluid (SF) leucocytes from patients with rheumatoid arthritis (RA).
Method: FURIN mRNA expression in PB and SF cells was determined by quantitative reverse transcription–polymerase chain reaction and FURIN plasma levels were measured using enzyme-linked immunosorbent assay. Associations between FURIN levels and demographic and clinical characteristics of the patients were determined.
Results: FURIN levels were significantly elevated in PB and SF mononuclear cells, T cells, and monocytes from RA patients compared to healthy controls. High FURIN levels were significantly associated with the prevailing prednisolone treatment, higher prednisolone doses, and increased C-reactive protein levels and Health Assessment Questionnaire values.
Conclusion: FURIN is significantly upregulated in RA PB and SF leucocytes, suggesting that it may have a role in the pathogenesis of RA. In addition, our results suggest that elevated FURIN expression is associated with the indicators of more severe RA.

In rheumatoid arthritis (RA), T cells, B cells, macrophages, and dendritic cells accumulate in the joints. Different cell types promote inflammation by secreting proinflammatory cytokines, such as tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ), granulocyte–macrophage colony-stimulating factor (GM-CSF), interleukin-1 (IL-1), IL-6, and IL-17 (1). These events ultimately lead to chronic inflammation, synovial hyperplasia and degradation of articular cartilage and bone (2).

FURIN was the first mammalian proprotein convertase (PCSK) enzyme to be discovered. FURIN belongs to the subtilisin superfamily of serine endoproteases and it converts immature proproteins into functional units (3). FURIN is essential for mammalian development and tissue homeostasis, but also contributes to the pathogenesis of several diseases, including cancers, elevated blood pressure, and atherosclerosis (4–6).

FURIN is important in the regulation of the immune system. Deleting FURIN in mouse T cells results in a loss of peripheral immune tolerance and aberrant T-helper cell polarization (7). In the myeloid cells, FURIN inhibits inflammatory responses by reducing the production of proinflammatory cytokines (8). The function of FURIN in immunoregulation is not entirely clear, but its expression in immune cells is critical for the functional maturation of anti-inflammatory pro-transforming growth factor-β_1 (pro-TGF-β_1) cytokine and the suppressive function of regulatory T cells (7).

Preliminary evidence also suggests that FURIN plays a role in the pathogenesis of human autoimmune diseases. In primary Sjögren's syndrome, an upregulated expression of FURIN has been detected in salivary gland biopsies (9) and in peripheral circulation (10). Previously, we found an association between a high plasma level of FURIN and a history of rheumatic disease in a prospective cohort study of patients with a suspected infection in the emergency room (11). We have therefore investigated FURIN levels in peripheral blood (PB) and synovial fluid (SF) leucocytes from RA patients. We also explored possible

*These authors contributed equally to this work
**These authors contributed equally to this work

Pia Isomäki, Department of Internal Medicine, Centre for Rheumatic Diseases, Tampere University Hospital, PO Box 2000, FI-33521 Tampere, Finland.
E-mail: pia.isomaki@uta.fi

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associations between FURIN levels and demographic and clinical characteristics of the patients.

Method

Patient samples

PB and SF samples were collected from patients with active RA. The characteristics of the patient cohorts are presented in Tables 1 and 2. In addition, purified T cells and monocytes were obtained from eight RA patients, with a median (range) age of 62 (37–87) years, duration of disease 18.5 (0.25–49) years, C-reactive protein (CRP) 25 (18–67.4) g/L, and erythrocyte sedimentation rate (ESR) 35.5 (10–91) mm/h. Control samples were obtained from healthy blood donors (Finnish Red Cross Blood Transfusion Service, Tampere).

All patients gave their written informed consent. This study was approved by the Ethical Committee of Tampere University Hospital, Tampere, Finland, and conducted according to the principles of the Declaration of Helsinki.

Cell preparations

Peripheral blood mononuclear cells (PBMCs) and synovial fluid mononuclear cells (SFMCs) were isolated by Ficoll-Paque Plus (Amersham Biosciences, Amersham, UK) density gradient centrifugation. T cells and monocytes were further purified from eight RA patients using magnetic beads (Miltenyi Biotec, Auburn, CA, USA). T cells were purified by negative selection (Pan T Cell Isolation Kit; Miltenyi Biotec) and monocytes were isolated by positive selection using anti-CD14-coated microbeads.

RNA isolation and quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis

Total RNA was isolated using the RNeasy MiniKit (Qiagen, Valencia, CA, USA). Total RNA was reverse transcribed using Maxima Reverse Transcriptase (Thermo Scientific, Waltham, MA, USA) and random hexamers (Thermo Scientific). The real-time PCR was carried out with the CFX96 instrument (Bio-Rad, Hercules, CA, USA) using a Maxima SYBR Green/ROX master mix (Thermo Scientific).

The following primers for FURIN and TATA-binding protein (TBP, housekeeping) were used: 5′-GAATATAATCCCAAGCGGTTTG-3′ and 5′-ACTTCACATCACAGCTCCCC-3′ for TBP and 5′-GGCAAAGCGACGGACTAAAC-3′ and 5′-CGTCCAGAATGGAGACCACA-3′ for FURIN.

Mean FURIN expression values from triplicate samples were obtained by dividing them by the mean values obtained for the TBP housekeeping gene.

Table 1. Associations of patients' characteristics and clinical parameters with FURIN mRNA levels in peripheral blood mononuclear cells (PBMCs) and synovial fluid mononuclear cells (SFMCs).

	FURIN mRNA in PBMCs			FURIN mRNA in SFMCs		
	n = 16	r	p	n = 8	r	p
Characteristics						
Age* (years)	66 (32–88)	0.0074	0.9784	54 (32–72)	−0.0238	0.9554
Gender (male)	7 (44)		0.7577	6 (75)		0.8571
Duration of disease (years)	15 (0–37)	−0.0517	0.8493	11 (1–37)	−0.4762	0.2329
Clinical parameters						
CRP (g/L)	30 (11–114)	0.3944	0.1306	44 (15–114)	0.9523	0.0003
ESR (mm/h)	42 (10–118)	0.0383	0.8881	32 (10–88)	0.6667	0.0710
SJC	7 (1–16)	0.3208	0.2258	7 (1–9)	0.3879	0.3423
TJC	6 (0–16)	0.2316	0.3880	3 (1–9)	−0.1473	0.7278
DAS28 (ESR)	5.3 (3.0–6.3)	0.2235	0.4053	4.8 (3.0–5.6)	0.4192	0.3013
VAS, global health	56 (0–100)	0.4341	0.0929	47 (1–90)	0.5714	0.1390
VAS, pain	58 (0–100)	0.3765	0.1506	47 (2–92)	0.5714	0.1390
HAQ	1.51 (0.00–2.50)	0.1550	0.5666	1.19 (0.00–1.74)	−0.2892	0.4873
RF positivity	10 (63)		0.1471	3 (38)		0.1429
Current treatment						
Prednisolone	12 (75)		0.0198	6 (75)		0.4286
Prednisolone dose (mg)	6.3 (0.0–15.0)	0.6809	0.0037	8.8 (0.0–25.0)	0.6747	0.0664
Methotrexate	7 (44)		0.6065	4 (50)		0.3429
DMARD	15 (94)		0.2500	7 (88)		1.0000
Biologics	2 (13)		0.2667	4 (50)		0.8857

Data are reported as median (range) or number (percentage).
The expression of FURIN mRNA was calculated in relation to the TATA-binding protein (TBP) expression (FURIN/TBP).
*Age at the time of sampling.
CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; SJC, swollen joint count; TJC, tender joint count; DAS28, 28-joint Disease Activity Score; VAS, visual analogue scale; HAQ, Health Assessment Questionnaire; RF, rheumatoid factor; DMARD, disease-modifying anti-rheumatic drug.

Table 2. Patients' characteristics and clinical parameters in all plasma samples and in two groups showing FURIN plasma levels below or above the detection threshold of the FURIN enzyme-linked immunosorbent assay (ELISA).

	All samples (n = 17)	FURIN < 123 pg/mL (n = 7)	FURIN ≥ 123 pg/mL (n = 10)	p
Characteristics				
Age* (years)	66 (52–88)	66 (58–77)	68 (52–88)	0.9056
Gender (male)	4 (24)	1 (14)	3 (30)	0.6029
Duration of disease (years)	7 (0–29)	7 (0–26)	8.5 (0–29)	0.9619
Clinical parameters				
CRP (g/L)	18 (0–68)	16 (0–39)	20 (0–68)	0.3772
ESR (mm/h)	40 (7–118)	40 (7–80)	41 (8–118)	0.5843
SJC	8 (1–15)	7 (1–9)	8 (4–15)	0.1633
TJC	6 (0–37)	6 (3–25)	5.5 (0–37)	0.9423
DAS28 (ESR)	5.6 (3.5–6.9)	5.5 (3.7–5.9)	5.7 (3.5–6.9)	0.2698
VAS, global health	63 (0–93)	56 (0–85)	72 (5–93)	0.3638
VAS, pain	55 (0–100)	54 (0–88)	64 (6–100)	0.5537
HAQ	1.25 (0.63–2.75)	0.75 (0.63–1.63)	1.74 (0.75–2.75)	0.0031
RF positivity	10 (59)	3 (43)	7 (70)	0.3500
Current treatment				
Prednisolone	13 (77)	4 (57)	9 (90)	0.2500
Prednisolone dose (mg)	7.5 (0.0–27.5)	5 (0.0–7.5)	10 (0.0–27.5)	0.0234
Methotrexate	8 (47)	3 (43)	5 (50)	1.0000
DMARD	12 (71)	5 (71)	7 (70)	1.0000
Biologics	4 (24)	1 (14)	3 (30)	0.6029

Data are reported as median (range) or number (percentage).
The p-values were derived from comparisons of the groups showing FURIN levels below or above the detection threshold of the FURIN ELISA.
*Age at the time of sampling.
CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; SJC, swollen joint count; TJC, tender joint count; DAS28, 28-joint Disease Activity Score; VAS, visual analogue scale; HAQ, Health Assessment Questionnaire; RF, rheumatoid factor; DMARD, disease-modifying anti-rheumatic drug.

FURIN enzyme-linked immunosorbent assay (ELISA)

FURIN plasma levels were measured using the Human Furin ELISA kit (Thermo Scientific). Duplicate determinations were carried out for each sample. The analysis was performed with a cut-off by dividing samples into those that were below the detection threshold (< 123 pg/mL) and those at or above it (≥ 123 pg/mL).

Statistical analysis

Statistical analyses were performed using SPSS Statistics version 22 (IBM Corp., Armonk, NY, USA). Fisher's exact test was used to test the relationship between two binomial variables. Analyses including ordinal and scale variables were conducted using the Mann–Whitney U-test (exact). The correlations for continuous variables were calculated using Spearman's correlation coefficient.

Results

Increased expression of FURIN in PB and SF T cells and monocytes from RA patients

First, we examined the levels of FURIN mRNA in PBMCs from patients with active RA and healthy controls. The expression of FURIN was significantly elevated in PBMCs from RA patients compared with healthy controls ($p < 0.001$) (Figure 1A).

To gain better insight into the cell populations which expressed increased levels of FURIN, we studied the expression of FURIN mRNA in isolated PB T cells and monocytes. As shown in Figure 1B and C, the levels of FURIN were elevated in both PB T cells and monocytes from RA patients compared to healthy controls, with monocytes expressing higher levels of FURIN than T cells.

In RA, SF T cells and macrophages are exposed to various inflammatory stimuli, and show an activated phenotype compared to the cells in PB. To investigate whether the cells from the inflamed joints express even higher levels of FURIN, SFMCs, SF T cells, and SF monocytes were isolated from patients with active RA presenting with arthritis of the knee joint. The levels of FURIN mRNA in SFMCs, T cells, and monocytes were comparable to those in RA PB cells (Figure 1A–C).

Finally, we also evaluated FURIN plasma levels in patients with RA and healthy controls. Plasma FURIN levels in both healthy controls and patients with RA varied considerably and no significant difference was observed between RA patients and healthy controls ($p = 0.23$) (Fig-Figure 1D). However, more patients (10 out of 17, 59%) than controls (five out of 14, 35%) demonstrated detectable levels of FURIN (≥ 123 pg/mL).

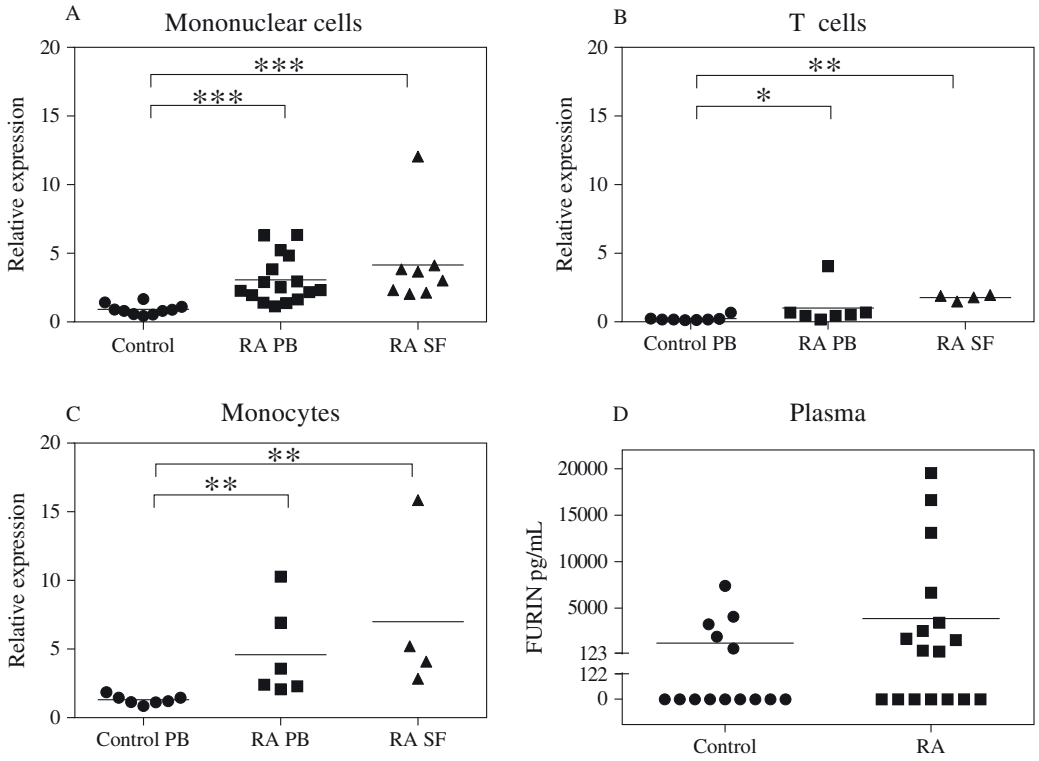

Figure 1. (A–C) FURIN mRNA expression and (D) FURIN plasma levels in patients with rheumatoid arthritis (RA) and healthy controls. Levels of FURIN mRNA were analysed by quantitative reverse transcription–polymerase chain reaction in: (A) peripheral blood (PB) mononuclear cells (n = 16) and synovial fluid (SF) mononuclear cells (n = 8); (B) T cells (n = 7 for PB and n = 4 for SF); and (C) monocytes (n = 6 for PB and n = 4 for SF) from healthy controls and patients with RA. Paired PB/SF samples were available from three patients in the mononuclear cell analysis, from four patients in the T-cell analysis, and from three patients in the monocyte analysis. Data are presented as relative FURIN expression divided by TATA-binding protein (TBP) level (A–C). (D) FURIN protein levels were measured by enzyme-linked immunosorbent assay in plasma samples from healthy controls and RA patients. Horizontal lines indicate the mean expression levels in each group. The detection limit of the assay was 123 pg/mL. Significant differences between study groups are marked with asterisks: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Correlation of FURIN levels with clinical characteristics of RA patients

We next examined whether FURIN mRNA levels in PBMCs or SFMCs correlated with any of the demographic or clinical characteristics of RA patients. We found a significant correlation between current prednisolone use ($p = 0.020$) and dose ($p = 0.004$) and FURIN mRNA levels in PBMCs. The expression of FURIN in SFMCs, in turn, showed a very strong correlation with the CRP level ($p < 0.001$) (Table 1). In addition, there were trends towards associations between FURIN level in SFMCs and ESR ($r = 0.6667$, $p = 0.0710$) and prednisolone dose ($r = 0.6747$, $p = 0.0664$).

We also examined whether FURIN levels in plasma were associated with any of the clinical characteristics. The Health Assessment Questionnaire (HAQ) disability index ($p = 0.003$) and prednisolone dose ($p = 0.023$) were significantly higher in the group demonstrating detectable levels of FURIN (Table 2). In addition, IL-1β, IL-2, IL-4, IL-6, IL-7, IL-10, IFN-γ, and TNF-α levels in plasma were determined in 10 patients and their levels were correlated with those of FURIN. The median levels of all cytokines studied were higher in the group demonstrating detectable levels of FURIN (n = 7) than in patients presenting with undetectable FURIN levels (n = 3; data not shown). However, this difference was significant only in the case of IL-2 (median 28 pg/mL vs 6 pg/mL, $p = 0.033$).

Discussion

The expression of FURIN in RA has not previously been systematically studied, and no data are available regarding FURIN expression in leucocytes from RA patients. Lin et al (12) reported upregulated expression of FURIN in synovial pannus derived from three patients with RA. In addition to RA, FURIN has been found to be upregulated in PBMCs and salivary gland biopsies in primary Sjögren's syndrome (9, 10). Elevated FURIN levels have

also been seen in patients with systemic lupus erythematosus (13). Together, these previous results and our current findings showing increased FURIN expression in RA indicate that FURIN is upregulated in different types of rheumatic disease.

Our results show that FURIN levels are associated with prednisolone treatment and dose, CRP levels, ESR, and HAQ disability index. Since prednisolone is used in the treatment of active RA, these findings suggest that FURIN is elevated mostly in patients with active and severe disease. This conclusion is supported by the fact that patients with elevated FURIN levels also showed global upregulation of plasma cytokines, particularly IL-2 associated with T-cell activation. These findings are also in line with our previous results indicating that FURIN expression is upregulated upon the activation of T cells and macrophages (5, 7). However, the inflammatory response in RA joints per se does not seem to result in further FURIN upregulation, since comparable levels of FURIN were observed in PB and SF samples.

Previous results suggest that FURIN has chiefly a protective role in arthritis. Systemic administration of FURIN was shown to ameliorate collagen-induced arthritis in mice (12). In addition, inhibition of FURIN was shown to enhance the invasive phenotype of synoviocytes from patients with RA (14). The protective role of FURIN in autoimmunity is also supported by previous results showing that FURIN is essential in maintaining peripheral immune tolerance (7) and that it inhibits proinflammatory cytokine production in myeloid cells (8). Notably, FURIN has been reported to inhibit matrix metalloproteinase-13 and to limit osteoarthritis in mice (15). The idea of using FURIN, its biologically active derivatives, or its inhibitors in the treatment of inflammatory diseases has been documented in patent applications (US20040127396A1 and WO2011144517A1).

Our current results suggesting that FURIN expression is associated with more active and severe RA are in line with previous data showing the upregulation of the anti-inflammatory cytokines IL-10 and TGF-β (1) in RA patients. The overexpression of these regulatory proteins may reflect an attempt to combat inflammation, but owing to the plethora of proinflammatory mediators present in RA, inflammation persists despite the actions of FURIN and other immunosuppressive proteins.

Conclusion

Taken together, our data demonstrate that FURIN is upregulated in severe RA. Further investigations are warranted to better determine the role of FURIN in the pathogenesis of RA and its potential value as a biomarker in inflammatory diseases.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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